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Association of thymosin β4 expression with clinicopathological parameters and clinical outcomes of bladder cancer patients

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The clinical significance of thymosin β4 (Tβ4) expression in bladder transitional cell carcinoma (BTCC) remains unclear. The present study assessed the relationship between the expression of Tβ4 protein and the clinicopathological features, as well as the prognosis of bladder cancer patients. Tβ4 protein expression in 24 normal bladder and 138 primary BTCC tissue specimens was detected by immunohistochemistry, and the association of this expression with BTCC clinicopathological features and recurrence as well as patient survival was analyzed. Tβ4 expression was significantly stronger in BTCC patients than in normal volunteers. The expression of Tβ4 was significantly associated with differentiation capability, tumor stage and lymph node metastasis ($P = 0.025$, 0.043, and 0.039, respectively). Moreover, Tβ4 expression was positively correlated with integrin-linked kinase (ILK) and β-catenin expression ($P = 0.042$, 0.031, respectively) and inversely correlated with E-cadherin expression ($P = 0.022$). In the present cohort of bladder cancer patients, Tβ4 expression was found to be a predictor of poor survival ($P < 0.05$); however, high Tβ4 expression exhibited unfavorable prognostic value for recurrence. These data suggested that Tβ4 is correlated with the pathogenesis of BTCC. In addition, the patients with higher Tβ4 expression had a shorter survival.

Key words: thymosin β4, bladder cancer, prognosis, immunohistochemistry, epithelial to mesenchymal transition, integrin-linked kinase, E-cadherin

Bladder transitional cell carcinoma (BTCC) is a prevalent global health problem and is the second most common malignancy of the urinary tract. Approximately 70% of all newly diagnosed cases present as superficial bladder cancer, the standard treatment for which is a transurethral resection (TUR) of the tumor [1]. However, approximately 60%-70% of these tumors recur, with 25% of these having progressed to a higher stage or grade [1]. Despite increased understanding of the molecular pathogenesis of bladder cancer, no reliable prognostic markers or risk factors are currently available for predicting the aggressive behavior or recurrence of bladder tumors, especially in *in situ* carcinoma and early-stage or low-risk tumors.

The epithelial to mesenchymal transition (EMT) is a process by which epithelial cells lose their polarity and convert into a mesenchymal phenotype [2, 3]. An emerging realization is that the progression of epithelial-derived tumors may also involve spatial or temporal occurrences of the EMT, which leads to cellular de-differentiation and loss of adhesive constraints, thereby providing a mechanism for carcinoma cells to acquire a more aggressive phenotype [4]. Furthermore, the EMT positively correlates with poor patient prognosis [5]. A hallmark of the EMT is the loss of E-cadherin expression, which is a very important component of the epithelial phenotype. Reduced E-cadherin expression is often correlated with tumor grade and stage and results in disrupted cell-cell adhesion as well as increased β-catenin in the nucleus [6,].

Thymosin β4 (Tβ4) is a 43-amino acid peptide that is a member of the beta-thymosin family; Tβ4 has been documented to be involved in a number of processes, including angiogenesis, wound healing, apoptosis, and inflammation [8, 9]. Recent studies have shown that Tβ4 is frequently overexpressed in malignant tumors and leads to increased tumor growth, metastasis and the EMT [10, 11]. Furthermore, the

presence of Tβ4 has been reported to facilitate cell migration and prostate cancer progression [12]. Tβ4 expression was shown to be significantly higher in metastasizing tumors than in non-metastasizing tumors and was associated with worse prognosis in early-stage, pulmonary non-small-cell carcinomas [13].

Our previous study [14] demonstrated that the EMT plays a central role in bladder cancer progression and metastasis. In addition, Tβ4 activity was a dominant factor in triggering the EMT of bladder cancer cells through upregulating ILK (integrin-linked kinase) expression and signal transduction, which led to a high level of β-catenin and a low level of E-cadherin. However, to date, no clinical data demonstrating the expression patterns of Tβ4 in bladder transitional cell carcinoma have been available.

In this study, we examined the expression patterns of Tβ4 in both clinical BTCC cases and normal bladders, and we investigated the relationship between prognostic histopathological characteristics and clinical outcome. Moreover, the same tumor groups were used to evaluate the correlation between the expression of Tβ4 and functionally related proteins, including ILK and β-catenin.

Patients and methods

Patients and tissue samples. All patients provided written, informed consent for the use of these clinical materials for research purposes, according to procedures approved by the ethics committee of our hospital. For the immunohistochemical assay, a total of 138 paraffin-embedded BTCC samples and 24 normal bladder tissue specimens (obtained more than 3 cm away from the tumor border) were collected from the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) between September, 2011 and December, 2014. All tissue samples were obtained from patients with histologically proven primary urothelial bladder cancer who had undergone TUR-B or partial or radical cystectomy. The clinicopathological data are summarized in Table 1. The patients included 106 males and 32 females, ranging in age from 41 to 88 years (mean age, 62.1 years). The regular follow-ups that were conducted consisted mainly of cystoscopic examination and pelvic computed tomography and were scheduled according to our standard protocol, i.e., every 3 months for at least 2 years, every 6 months for the next 3 years, and then every 12 months for life. Survival time was calculated from the date surgery was performed to the date of death or the date of the last follow-up. The follow-up period was closed on Apr 30, 2015. The mean follow-up period was 29.8 months (range, 5-41 months). The grade and stage of the patients were defined according to the 2004 WHO criteria for grade and the 2009 TNM classification system [15, 16].

Immunohistochemistry. Tβ4, ILK, β-catenin, and E-cadherin expression levels in all paraffin-embedded tumor specimens were detected using an immunohistochemical method. The tissue sections were incubated overnight with monoclonal antibody at a 1:100 dilution and were then treated with a streptavidin-peroxidase immunohistochemical staining kit (Zymed Laboratories Inc., CA, USA), according to the manufacturer's protocol. Samples incubated with normal serum instead of primary antibodies were used as negative controls. Finally, depending on the marker, the different distributions were scored as membranous, cytoplasmic, and/or nuclear.

Immunohistochemistry scores. Three well-trained and blinded observers examined the slides and scored the Tβ4, ILK, β-catenin and E-cadherin expression levels. Each slide was evaluated for Tβ4, ILK, β-catenin and E-cadherin immunoreactivity using a semi-quantitative scoring system for both the intensity of the stain and the percentage of positive neoplastic cells. Stained Tβ4 and ILK deposits in the cytoplasm, β-catenin deposits in the nucleus and E-cadherin deposits in the membrane were considered positive neoplastic indicators. The intensity of positive staining was scored as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The extent of positive staining was scored according to the percentage of positive cells in the respective lesions: 0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. For the purpose of statistical evaluation, tumors with a final staining score of >3 were considered to exhibit high expression. The median of this series (i.e., an intensity score of ≥2 with at least 26% positively stained cells) was used as a threshold for distinguishing tumors with low (<25%) or high (>25%) levels of Tβ4, ILK, β-catenin and E-cadherin. These criteria were based on previously published reports [17, 18].

Statistical analysis. SPSS, version 13.0 (SPSS Inc., Chicago, IL, USA), was used for all statistical analyses. The relationships between Tβ4 or E-cadherin expression and each clinicopathological parameter were evaluated using a Chi-square test or Fisher's exact test. Patients who died from other diseases were excluded from the survival analysis. Overall survival curves were obtained by the Kaplan–Meier method, and differences between the survival curves were examined using the log-rank test. The Cox proportional hazards model (Cox regression test) was used for multivariate analyses. All reported P values are two-sided, and P values < 0.05 were considered statistically significant. The correlation between Tβ4 and ILK/β-catenin expression was assessed using the Spearman test, and $P < 0.05$ was considered statistically significant.

Results

Expression of Tβ4, ILK, β-catenin and E-cadherin in BTCC tissue samples. Immunohistochemistry was used to investigate Tβ4 protein expression in 24 normal bladder tissue specimens and 138 paraffin-embedded BTCC tissue samples that represented different stages of bladder cancer. As shown in Figure 1, positive Tβ4 immunostaining was predominantly observed in the cytoplasm of carcinoma cells at significantly higher levels than those in normal cells; however, E-cadherin staining was mainly observed in the membranes of normal

Table 1. Correlation between Tβ4/E-cadherin expression and clinicopathological characteristics of bladder cancer patients

Variable	Total	Tβ4		*P*-value	E-cadherin		*P*-value
		Low expression[a]	High expression[b]		Low expression[a]	High expression[b]	
All cases	162						
Normal	24	20	4		1	23	
BTCC	138	41	97	0.027*	106	32	0.018*
Age (years)							
≤60	59	19	40		43	16	
>60	79	22	57	0.584	63	16	0.344
Gender							
Male	106	32	74		77	29	
Female	32	9	23	0.821	29	3	0.055
Tumor size (cm)							
≤3	90	25	65		61	29	
>3	48	16	32	0.505	45	3	0.078
Number of tumors							
Unifocal	51	19	32		39	12	
Multifocal	87	22	65	0.058	67	20	0.940
T stage							
Tis	9	2	7		6	3	
Ta//T1	61	30	31		50	11	
T2/T3/T4	68	9	59	0.043*	51	17	0.625
Grade							
G1	36	28	8		12	24	
G2-G3	102	13	89	0.025*	94	8	0.023*
Lymph node metastasis							
N0	78	26	52		56	22	
N1/N2/N3	60	15	45	0.039*	50	10	0.113

[a]Low expression: staining score < 3; [b]High expression: staining score ≥ 3; *$P < 0.05$

epithelial cells. Positive staining for ILK and β-catenin was partially observed in not only the cytoplasm and nuclei of tumor cells but also in intraglandular deposits within cancerous lesions (Figure 1).

Correlation between Tβ4/E-cadherin protein expression and clinicopathological features. In the current study, 34 patients (24.6%) had a poorly differentiated BTCC, 68 patients (49.3%) had moderately differentiated BTCC, and 36 patients (26.1%) had well differentiated BTCC. The association between Tβ4/E-cadherin protein expression and the clinicopathological features of BTCC was examined. As shown in Table 1, high levels of Tβ4 protein expression were significantly correlated with tumor grade and stage, as well as lymph node metastasis ($P = 0.025$, 0.043, and 0.039, respectively). However, Tβ4 protein expression was not associated with other clinicopathological features, such as age, sex, tumor size, and number of tumors. In contrast, E-cadherin protein expression was not only found significantly more often in the normal group than in the BTCC group ($P = 0.018$) but also was significantly correlated with tumor grade ($P = 0.023$).

High ILK, high β-catenin and low E-cadherin expression levels are associated with high Tβ4 expression in patients with advanced-stage BTCC. Tβ4 expression was significantly positively correlated with ILK and β-catenin expression ($P = 0.042$, 0.031, respectively), and E-cadherin expression was lower in the high-level Tβ4 BTCC tissues than in the low-

Table 2. Relationship between Tβ4 and ILK/β-catenin expression

Variables	Tβ4		*P*-value
	Low expression[a] (%)	High expression[b] (%)	
ILK			
Low expression (%)	31/41 (75.61)	28/97 (28.87)	
High expression (%)	10/41 (24.39)	69/97 (71.13)	0.042*
β-catenin			
Low expression (%)	26/41 (63.41)	32/97 (32.99)	
High expression (%)	15/41 (36.59)	65/97 (67.01)	0.031*
E-cadherin			
Low expression (%)	16/41 (39.02)	90/97 (92.78)	
High expression (%)	25/41 (60.98)	7/97 (7.22)	0.022*

[a]Low expression: staining score < 3; [b]High expression: staining score ≥ 3; *$P < 0.05$

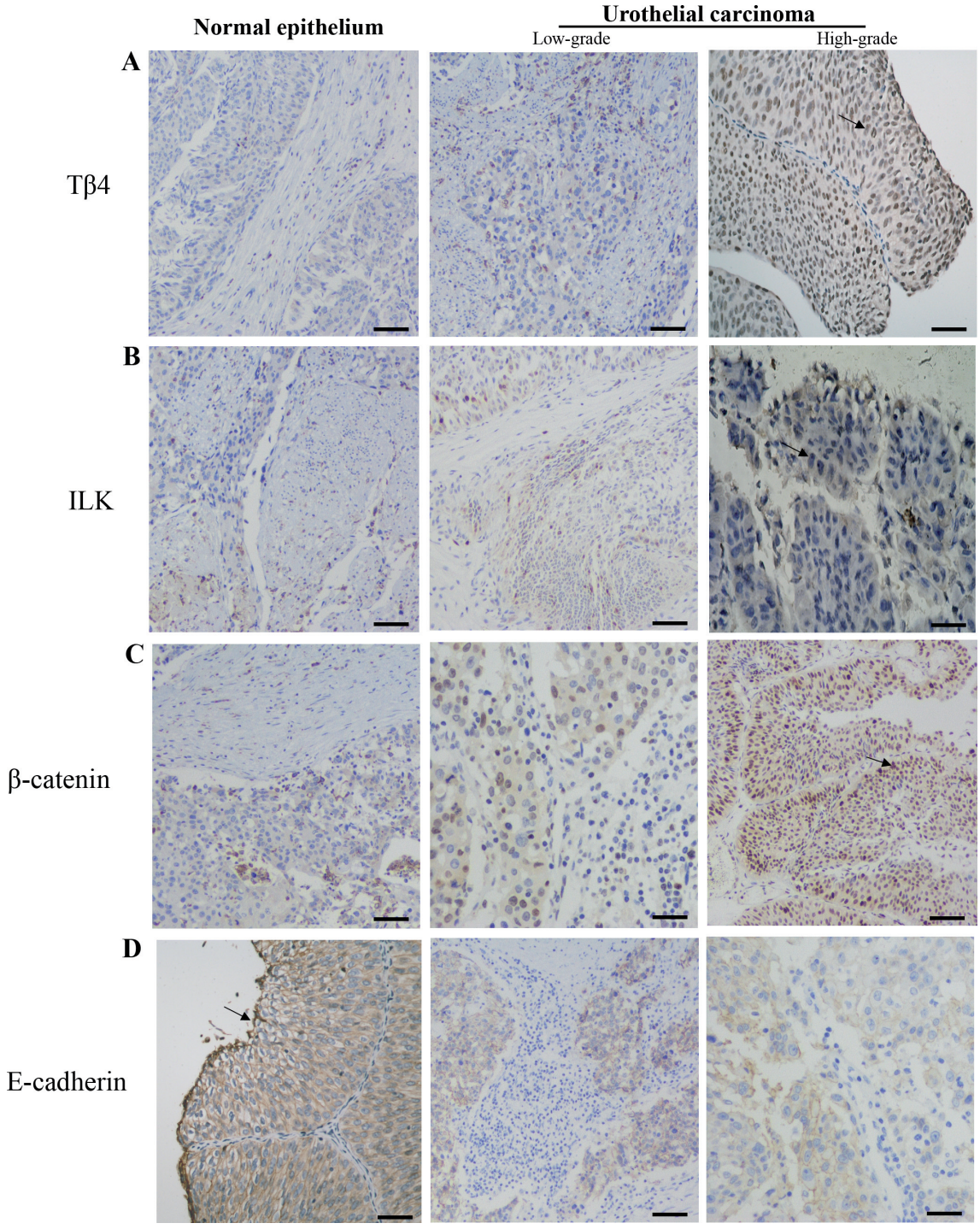

Figure 1. Immunohistochemical staining of EMT markers. (A), Positive cytoplasmic Tβ4 expression in high-grade BTCC tissue samples (arrow). (B) Positive cytoplasmic ILK expression in high-grade BTCC tissue samples (arrow). (C) Positive nuclear β-catenin staining in high-grade BTCC tissue samples (arrow). (D) Positive membranous E-cadherin expression in normal bladder urothelium (arrow). Bars represent 50 μm.

level Tβ4 BTCC tissues ($P = 0.022$) (Table 2). Interestingly, we found these strongly positive correlations between Tβ4 and ILK/β-catenin expression levels more often in highly invasive tumors; however, no significant correlations were observed between the expression levels of each protein in less-invasive tumors (data not shown).

Impact of Tβ4 protein expression on the prognosis of BTCC patients. The Kaplan–Meier and log-rank tests that

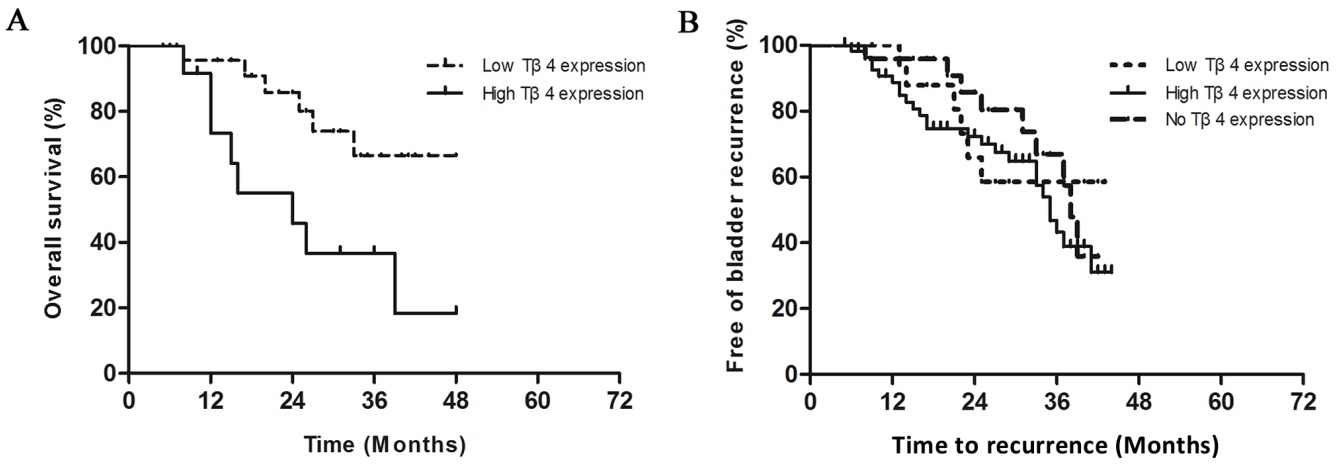

Figure 2. Kaplan-Meier analysis of overall survival (A) and recurrence-free survival (B) in all bladder cancer patients according to Tβ4 expression.

were used for univariate analyses revealed statistically significant differences in the overall survival of BTCC patients who did and did not have high Tβ4 expression (P = 0.035) (Figure 2A). The log-rank test further demonstrated that advanced pathological stage and lymph node metastasis were closely correlated with shorter survival (P = 0.015, 0.038, respectively) (Table 3). In addition, a multivariate analysis using the Cox proportional hazard regression model revealed Tβ4 level, pathological stage and lymph node metastasis as independent prognostic factors of overall survival (Table 4). As other EMT biomarkers had no influence on survival in the univariate tests, we did not perform further multivariate Cox-regression analyses.

The total of 52 (37.68%) patients experienced recurrence during the observation period: one recurrence was observed in 35 (25.36%) patients; two recurrences were observed in 10 (7.25%) patients; and three or more recurrences were observed in 7 (5.07%) patients. The univariate and multivariate analyses of recurrence risks showed that the number of tumors, the degree of tumor invasion and tumor grade are the independent prognostic factors that affected recurrence in the BTCC patients (Table 3, Table 4). Although Tβ4 expression was not a significant prognostic factor for BTCC recurrence, high Tβ4 staining intensity was observed more often in tumors from patients with than without recurrence/metastasis, as shown in Figure 2B.

Table 3. Univariate analysis of risk factors for overall survival and recurrence in patients with BTCC

Variable	Survival			Recurrence		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (years)						
≤60 versus >60	1.312	0.921-1.862	0.141	0.961	0.511-1.827	0.902
Gender						
Male versus Female	1.043	0.705-1.557	0.852	1.393	0.792-2.472	0.264
Tumor size (cm)						
≤3 versus >3	1.063	0.741-1.529	0.753	0.945	0.496-1.823	0.922
Number of tumors						
Unifocal versus Multifocal	1.094	0.772-1.567	0.625	3.013	2.725-3.362	0.024
T stage						
Ta/Tis/T1 versus T2/T3/T4	4.864	2.833-5.644	0.015	4.684	3.656-5.163	0.013
Lymph node metastasis						
N0 versus N1/N2/N3	3.325	2.863-4.037	0.038	1.476	1.053-1.954	0.137
Grade						
G1 versus G2 and G3	0.947	0.641-1.398	0.916	2.657	2.365-3.186	0.041
Tβ4 expression						
Low versus high	3.751	3.045-4.352	0.035	1.281	0.992-1.488	0.454

HR, hazard risk; 95% CI, 95% confidence interval

Table 4. Multivariate analysis of prognostic factors for overall survival and recurrence in patients with BTCC

Variable	Survival			Recurrence		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Number of tumors						
Unifocal versus Multifocal				2.883	2.705-3.596	0.033
T stage						
Ta/Tis/T1 versus T2/T3/T4	3.784	2.806-4.112	0.029	4.484	3.556-4.963	0.019
Lymph node metastasis						
N0 versus N1/N2/N3	2.815	2.603-3.154	0.047			
Grade						
G1 versus G2 and G3				2.515	2.235-2.986	0.044
Tβ4 expression						
Low versus high	3.022	2.901-3.327	0.034			

HR, hazard risk; 95% CI, 95% confidence interval

Discussion

Recurrence and distant metastasis are frequently observed in BTCC patients, and the 5-year survival rate for high-grade tumors remains quite low. Therefore, discovering novel molecular targets that may be used in the development of more effective therapies for BTCC patients is crucial.

Numerous *in vitro* and *in vivo* studies have suggested that the EMT is associated with cancer cell invasion and metastasis in various malignancies, including BTCC. In this study, we first examined the relationship between Tβ4 and E-cadherin expression and the clinicopathological features of BTCC. Our findings revealed that the expression rate of Tβ4 in BTCC and normal bladder tissue was 70.3% and 16.7%, respectively. High Tβ4 expression was found in BTCC tissue, supporting our previous finding of high Tβ4 mRNA expression in BTCC tissue. Furthermore, there was a trend of increasing Tβ4 expression with both increasing tumor grade and stage. Though high Tβ4 expression was not observed more often in non-muscle-invasive bladder urothelial carcinomas (Ta/Tis/T1), our results revealed a slightly increase in the expression of Tβ4 in the patients with stage Tis (7/9). This finding is possibly due to the fact that urothelial CIS is always high-grade, which is strongly associated with aggressive bladder cancer behavior, and high Tβ4 expression in carcinoma *in situ* favours tumour growth and invasion. Moreover, increased Tβ4 expression has also been correlated with lymph node metastasis. Our data show a statistically significant association between Tβ4 overexpression and poor prognostic histopathological features, as well as clinical BTCC aggressiveness. In contrast with Tβ4, E-cadherin expression was decreased in BTCC tissue. In addition, while E-cadherin expression was associated with the degree of BTCC tissue differentiation ($P = 0.023$), it was not associated with other clinical pathological features ($P > 0.05$). More importantly, while Tβ4 expression was detected with lower sensitivity in low-grade tumors, it was reliably detected in high-grade tumors, with a sensitivity of up to 100% (data not shown). These results indicate that under the circumstances of this study, Tβ4 had limited potential as a marker for the early detection of urothelial cancer.

Tβ4 may provide a mechanism for carcinoma cells to acquire a more aggressive phenotype through inducing the EMT by activating ILK and β-catenin, as well as abolishing E-cadherin function [11]. This study also examined the expression patterns of Tβ4 and other proteins involved in the EMT in BTCC tissue, including ILK and β-catenin. The expression of Tβ4, ILK and β-catenin were weak during the early stages (Ta to T1) of BTCC but were significantly increased from stage T2 onward. The immunohistochemistry results demonstrated that ILK/β-catenin expression was significantly higher in samples with high rather than low Tβ4 expression; in addition, a positive correlation was found between Tβ4, ILK and β-catenin expression in the BTCC samples. Furthermore, high Tβ4 expression was detected in 90 of 97 cases with low E-cadherin expression in tumor tissues, and Spearman's analysis revealed that E-cadherin expression was negatively correlated with Tβ4 expression in BTCC tissues ($P = 0.022$), as shown in Table 2. Consistent with the results of previous studies, these findings suggest that Tβ4 can play an important role in BTCC aggressiveness through factors such as ILK and β-catenin and may trigger early-stage tumor invasion and metastasis.

The multivariate analyses indicated that TNM stage and lymph node status were exclusively independent prognostic factors for BTCC patients with high Tβ4 expression, which was in accordance with a previous observation that TNM stage and lymph node status were the most common prognostic factors of malignant tumors.[16] Moreover, positive Tβ4 staining was consistently found in locally invasive, high-grade BTCC, and patients with higher Tβ4 expression survived for a shorter time. These results may be partially explained by the EMT being an event that takes place early in tumor progression [19]. Furthermore, once tumor cells infiltrate a distant organ, they undergo a reverse EMT transition and regain their epithelial phenotype along with increased self-renewal and proliferative capacity [20]. Therefore, although the EMT markers tested

in this study failed to predict long-term outcomes in our carcinoma cohort, they may be valuable indicators of locally invasive cancer.

Past studies of bladder cancer have demonstrated that the EMT is closely associated with grave clinical characteristics, such as recurrence and poorer survival [21]. However, after 5-41 months of active surveillance, data revealed that there were no significant differences in Tβ4 expression between recurring and non-recurring tumors ($P = 0.454$) (Table 3). Probable explanations for this observation include the following: [1] In our study, while lower Tβ4 expression was observed in 70 patients with Ta/Tis/T1 tumors compared with that found in patients with stage T2/T3/T4 tumors ($P < 0.05$), in many cases, non-muscle-invasive bladder cancer has high recurrence due to low progression rates and long-term survival; in addition, patients with muscle-invasive bladder cancer are at higher risk for cancer-specific mortality [22]. The degree of invasion and number of tumors are the most important prognostic factors for the recurrence of non-muscle-invasive bladder cancer [23]. Nevertheless, in this study, there was no significant association between Tβ4 and the number of tumors. One potential mechanism underlying this observation is that high Tβ4 expression mainly accelerates the deteriorative process via specific EMT-inducing signals in advanced BTCC, thereby inducing local invasion and distant metastasis; however, the precise details of how this process directly affects recurrence in early-stage carcinoma remains unknown. In addition, E-cadherin is not only the first and most important regulator of the EMT but also is clearly associated with the clinical outcomes of BTCC, such as recurrence [24]. However, several factors (e.g., microRNA-205, Twist) or pathways (e.g., epidermal growth factor receptor, tumor necrosis factor-related apoptosis-inducing ligand) might interact with Tβ4 or influence the biological features of BTCC through acting on E-cadherin expression [25]; as a result, our findings demonstrated that Tβ4 was likely not an independent prognostic factor for recurrence.

Routine lymphadenectomy was also performed in patients who underwent radical cystectomy, and the results of both univariate and multivariate analyses demonstrated that lymphatic metastasis positively impacted overall survival.

In conclusion, our study clarified the correlation between Tβ4 expression and clinicopathological parameters of BTCC, as well as the prognostic value of Tβ4 protein expression in advanced BTCC. Although our results are promising, our study has several limitations. Further functional analyses using well-defined models and more elaborate approaches will not only improve our understanding of Tβ4 but also contribute to the development of novel diagnostic and therapeutic strategies against bladder cancer.

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