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ORIGINAL ARTICLE

Expression of transcription factor Twist1 in bladder urothelial carcinoma and its clinical significance

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Summary

Purpose: Transcription factor Twist1 is known to play a vital role in cancer development, progression and metastasis. However, regulation mechanisms beneath Twist1 expression, as well as the correlation between its expression and bladder urothelial carcinoma (BUC), are still under investigation. Herein, we tried to investigate the expression of Twist1 in BUC specimens and non-cancerous mucosas and illustrate their relationships with clinicopathological features.

Methods: The expression of Twist1 mRNA in 42 fresh BUC specimens and 13 paired non-cancerous mucosas was detected by real-time fluorescence quantitative reverse transcription polymerase chain reaction (RFQ-RTPCR). Immunohistochemistry (IHC) was used to detect the expression of Twist1 protein in 40 paraffin embedded BUC specimens and 14 paired non-cancerous mucosas, and their relationships with clinicopathological features.

Results: The expression levels of Twist1 mRNA in 13 paired BUC specimens were significantly lower than the non-cancerous mucosas. The positive expression rate of Twist1 protein in BUC specimens (90.0%; 36/40) was significantly higher than the non-cancerous mucosas (7.14%; 1/14). Twist1 protein was mainly distributed in the nucleus, and expressed obviously in the mesenchymal cells of several specimens (13.9%;5/36). However, expressions of Twist1 protein were not associated with TNM stage and grade. It was also shown that the expression tendency of Twist1 protein was distinct from Twist1 mRNA, and both were not correlated with age, gender, and smoking history.

Conclusion: As a probable potential biomarker for BUC, Twist1 gene may play a role as an oncogene during the tumorigenesis and development of BUC. Its abnormal protein expression may be associated with disordered regulations after transcription.

Key words: bladder cancer, gene expression, immunohistochemistry, Twist1

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Introduction

As a highly conserved basic helix-loop-helix (bHLH) transcription factor, Twist1 was originally identified as a key inducer of mesoderm formation in Drosophila, and plays a vital role in the regulation of cell migration [1]. It's now well accepted that Twist1, which may function as an oncogene during tumorigenesis and development of cancer, promotes tumor invasion and migration through inducing the epithelial-mesenchymal transition (EMT) of cancer cells. Moreover, Twist1 is also involved with cell apoptosis, chemoresistance, angiogenesis and the generation of cancer stem cells (CSCs) [2,3]. Recently, upregulation of Twist1 protein has been reported in several cancers, such as liver [4], lung [5], stomach [6], breast [7] and bladder [8-11], and its expression levels were found to be associated with tumor progression, metastasis and poor prognosis.

Several studies have reported that expression of Twist1 protein was not correlated with its mRNA levels [12-14], a fact that was explained by post-transcriptional regulations. However, until now, the association between Twist1 protein and mRNA expression has not been established in human cancers, including BUC. Herein, we used RFQ-RTPCR and IHC to investigate the expression of Twist1 mRNA and protein in BUC tissues and paired non-cancerous mucosas, respectively. Furthermore, we tried to describe their relationships with clinicopathological features and elucidate the association between Twist1 and BUC.

Methods

Patients

The present study was based on a consecutive series of patients undergoing surgery for BUC (transurethral resection or cystectomy) at the Department of Urology, The First Affiliated Hospital of Xiamen University, Xiamen, China. All specimens were obtained immediately after surgery, including fresh specimens and/or formalin-fixed, paraffin-embedded specimens (not all the specimens were paired). Finally, a total of 42 fresh BUC specimens and 13 paired non-cancerous mucosas (taken at least 3 cm from the outer tumor margin) were acquired, freezed in liquid nitrogen and stored at -80°C until processing. We also obtained 40 paraffin-embedded BUC specimens and 14 paired non-cancerous mucosas. All the original slides of the specimens were reviewed for histopathological staging (2002 UICC TNM classification) and grading (World Health Organization classification) by two independent pathologists. Considering that the incidence of human BUC increases especially after 50 years of age, we took the 50 years as boundary of age groups. Smoking condition of patients was evaluated by the smoking index (SI: cigarettes per day × smoking years), and divided into non-smoking group (SI<1) and smoking group (SI≥1). All patients in the study gave written informed consent whilst none had received chemoradiotherapy or immunotherapy before surgery. Clinicopathological characteristics of all the informative specimens are shown in Tables 1 and 3. The noninformative samples included unrepresentative samples or fresh samples with degradated RNA.

RNA extraction and RT-PCR

Total RNA was extracted from malignant and nonmalignant samples with the RNA simple Total RNA Kit (Tiangen Inc., Beijing, China), according to the manufacturer's protocol. The RNA extracted was quantified with a Shimadiu UV-200 Spectrophotometer and stored at -80°C until processing. For reverse transcriptase RT-PCR, cDNA was synthesized from 500 ng RNA with the PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa Code DRR037S, China).

RFQ-RTPCR

The cDNA was then amplified by SYBR Premix Ex Taq™ (Perfect Real Time, TaKaRa Code DRR041, China), using an Light Cycler 480 analyzer (Roche, Switzerland), with the following primers: Twist1[2] - 5'-GGAGTCCGCAGTCTTACGAG - 3' and 5'- TCTGGAGGACCTGGT AGAGG – 3', Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) - 5'- GAAGGTGAAGGTCGGAGTC- 3' and 5' - GAAGATGGTGATGGGATTTC - 3'. The PCR conditions: 45 cycles of 95°C for 5 sec, 56°C for 20 sec, and 72°C for 20 sec. The initial denaturation step was performed at 95°C for 30 sec. At the end of the PCR cycles, melting curve analyses were performed to confirm the generation of the specific expected PCR product (95°C for 5 sec and 65 °C for 1 min). In the end, the relative expression of Twist1

mRNA was presented as a $2^{-\Delta Ct}$ value ($\Delta Ct = Ct_{Twist1} - Ct_{GAPDH}$), and PCR products were electrophoresed on 2% agarose gels to further identify the specificity.

Immunohistochemistry staining

IHC studies were performed using 2-step EliVision™ plus kit and diaminobenzidene (DAB) (Maixin, Fuzhou, China, Code No: KIT-9901& DAB-0031) to detect Twist1 protein expression. The paraffin slides (4μm thick) were deparaffinized and rehydrated with xylene and serial ethanol dilutions. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min, followed by antigen retrieval (boiling-treated in autoclave with 10-mmol citric buffer with pH 6.0 for 2 min). Slides were incubated for 1 h at room temperature with the Abcam mouse monoclonal Twist1 antibody (1:100) (Twist2C1a, Code No: ab50887). Phosphate buffered saline (PBS) substitute for Twist1 antibody served as negative control.

The 2-way scoring system [11] was used for analysis of Twist1 results in this study with modifications. First, we scored the staining intensity in 4 degrees: negative=0; weak=1; moderate=2; and strong=3. Then, we estimated the proportion of positive cells with the following criteria: ≤5%=0; 6-25%, 1; 26-50%=2; and ≥51%=3. Subsequently, the scores were added up and divided into 4 groups (≤1, 2-3, 4-5, and 6) as 4 corresponding staining degrees (-, +, ++, +++).

Statistics

All statistical analyses were performed using a SPSS 13.0 software (SPSS, Chicago, IL). Normal distribution was first tested using Shapiro-Wilk test. For RFQ-RTPCR data, Mann-Whitney U test, Wilcoxon Signed Rank test (Wilcoxon test), and Kruskal Wallis test were used. The Pearson x^2 test, correction x^2 test and Fisher's Exact test were used for IHC data. Statistical significance was put at two-sided $p< 0.05$.

Results

Relative Twist1 mRNA expression

The results of RFQ-RTPCR showed that melting peaks of Twist1 and GAPDH were simple (Figure 1), and the expression levels of Twist1 mRNA in 13 paired BUC specimens were significantly lower than the non-cancerous mucosas (median 1.47 and 4.05, respectively; $p<0.01$, Wilcoxon test) (Figure 2).

As the numbers of each stage BUC specimens were small, we combined T_a and T_1 stages as the superficial bladder cancer group (T_{a-1}), and T_2, T_3 and T_4 stages together as the invasive bladder cancer group (T_{2-4}). Twist1 mRNA expression in T_{a-1} group was about twice as much than in T_{2-4} group ($p<0.05$, Mann-Whitney test), and its expression levels in grade (G) 3 group were significantly different from G1 and G2 ($p<0.05$, Mann-Whitney test). However, there was no difference between G1 and G2 groups ($p>0.05$, Mann-Whitney test). Furthermore, Twist1 mRNA expression was not correlated to age, gender and smoking history ($p>0.05$, Mann-Whitney test) (Figure 2, Table 1).

Immunohistochemical analysis of Twist1

The positive expression rate of Twist1 protein in BUC specimens (90.0%, 36/40) was significantly higher than in the non-cancerous mucosas (7.14%;1/14) ($p<0.001$, Pearson x^2 test). Twist1 protein was mainly distributed in the nucleus; the percentages of BUC samples expressing the Twist1 protein either in the nucleus or in the cytoplasm were 55.6% (20/36) and 33.3% (12/36), respectively (Figure 3A,C,E). The difference between nuclear and cytoplasmic protein expression was not associated with clinicopathological features ($p>0.05$, Fisher's Exact test, Table 2). Interestingly, Twist1 protein was expressed in the mesenchymal cells of several BUC specimens (13.9%;5/36) (Figure 3D). Unexpectedly, the expressions of Twist1 protein were not associated with TNM stage, grade, age, gender and smoking history ($p>0.05$, Table 3).

Discussion

Two Twist genes, Twist1 (Twist) and Twist2 (Dermo-1), exist in vertebrates sharing more than 90% of identity in the carboxy-terminal domains of their proteins [15]. The human Twist1 gene located at chromosome 7p21.2 encodes the Twist1 protein consisting of 202 amino acids, which acts as a transcription factor through the pattern of dipolymer. Previously, a research [2] on breast cancer found that the transcription factor Twist1 promoted tumor invasion and metastasis through induc-

Table 1. Relationship between the mRNA expression of Twist1 and clinicopathological features

Groups		$2^{-\Delta\Delta ct}\times 10^{-3}$ *(Percentiles)*			*p-value*
	N	*25%*	*50%*	*75%*	
T stage					0.011
T_{a-1}	27	1.61	3.19	4.50	
T_{2-4}	15	0.56	1.37	2.63	
Grade					0.023[a]
1	9	1.52	1.91	4.62	
2	27	1.26	2.65	4.27	
3	6	0.37	0.85	1.48	
Age (years)					0.321
≤50	13	1.44	2.65	4.18	
>50	29	1.15	1.67	3.88	
Gender					0.666
Female	6	1.04	1.30	5.87	
Male	36	1.39	2.12	3.86	
Cigarette smoking					0.875
SI<1	11	1.16	1.68	5.30	
SI≥1	31	1.37	1.91	3.85	

[a]Kruskal Wallis test. Multiple comparisons (Wilcoxon test:G1 and G2, p=0.898; G1 and G3, p =0.010; G2 and G3, p=0.012 (2-tailed). SI: smoking index

tion of EMT in cancer cells. Loss of E-cadherin appears to be critical to an EMT. Similar with Snail or Slug, Twist1 inhibited E-cadherin expression at the transcriptional level through binding to E-boxes elements of the E-cadherin promoter. Twist1 mRNA and protein were both increased in hepatocellular carcinoma as compared with non-cancerous tissues, and the upregulated Twist1 protein was associated with metastasis and poor prognosis [4]. Recently, an article about 151 colorectal cancer cases demonstrated that Twist1 expression was restricted to tumor tissues and correlated with lymph node metastasis and overall survival (OS) [16].

In our study, Twist1 protein was significantly upregulated in BUC tissues compared to non-cancerous mucosas, but without any correlation with TNM stage or grade of malignancy, indicating its potential importance in the tumorigenesis and development of BUC, especially in early stage. In addition, we found Twist1 protein was mainly expressed in cell nuclei without any association with clinicopathologic data, suggesting that Twist1 protein may regulate the cellular life activities and promote cancer development through pathways of nuclear translocation. A previous study [11] with 164 bladder cancer tissues, 37 nonmalignant bladder tissues and 25 matched lymph node metastatic lesions, also showed that the positive rate of Twist1 protein was significantly elevated in cancer tissues(>90%)compared with nonmalignant tissues (about 20%). However, the authors found that Twist1 protein expression was found mainly in the cytoplasm and was associated with cancer progression. Yet, in recent years, several studies on bladder cancer found that the positive rate of Twist1 protein was about only 40% in tumor tissues [8-10], and its expression was associated with smoking history [10]. Particularly, Gort et al. also described nuclear Twist1 staining in malignant cells, and found no correlation between Twist1 expression and clinicopathologic data [12]. Certainly, more studies with larger sample size are needed to confirm our present findings.

Simultaneously, we found strong positive nuclear expression of Twist1 protein in the stromal compartment of several BUC tissues, where there exist two cell populations (epithelial-mesenchymal trans-

formed tumor cells and stromal fibroblastic cells). Twist1 expression might be activated in these cells due to growth factors produced by the tumor. However, expression of Twist1 protein in the stromal compartment was not associated with prognosis [7]. In another study, Twist1 expression was especially observed in gastric cancer-associated fibroblasts, and was associated with disease progression and poor patient survival [17]. Obviously, more clinical studies and follow up data are needed to validate the expression sites of Twist1 protein, as well as its correlation with tumor progression and patient prognosis.

We found that Twist1 mRNA expression in non-cancerous bladder mucosas was significantly higher than paired cancer tissues, and its expression in T_{a-1} stage group was higher than in T_{2-4} stage group. These findings implied that the pre-transcriptional regulations, such as DNA promoter methylation, might be involved in suppressing Twist1 mRNA expression. In accordance with other studies [12,18], a detection base on 91 BUC tissues and 39 non-cancerous bladder mucosas found that the promoter methylation level of Twist1 in cancer tissues was significantly higher than in non-cancerous tissues [19]. Furthermore, the hypermethylation frequencies of Twist1 in cervical carcinoma increased progressively along with tumorigenesis and cancer development [18]. Although regulations before transcription seem to explain our results, there was no evidence to support relevance between Twist1 promoter methylation and mRNA expression until now. Gort et al. considered other signal pathways involved in the regulations [12]. Indeed, we need more basic research with *in vitro* models to further elucidate the intrinsic regulation mechanisms of Twist1 mRNA.

In this study, the trend of Twist1 protein expression was discordant with mRNA expression, suggesting that Twist1 protein might be regulated mainly through post-transcriptional actions. Similarly, no correlation between Twist1 protein and mRNA ex-

Table 2. Correlation between Twist1 protein expression positions and clinicopathological features

		Twist1 expression positions		
Groups	*Positive Num[a]*	*N1[a]+B/2[b] (%)*	*N2[a]+B/2β[b](%)*	*p-value*
T stage				0.471
T_{a-1}	24	15+1 (66.7)	7+1 (33.3)	
T_{2-4}	12	5+1 (50)	5+1 (50)	
Grade				0.054
1	7	5 (71.4)	2 (28.6)	
2	23	9+2 (47.8)	10+2 (52.2)	
3	6	6 (100)	0 (0)	
Age (years)				0.706
≤50	10	7 (70)	3 (30)	
>50	26	13+2 (57.7)	9+2 (42.3)	
Gender				1.000
Female	5	3 (60)	2 (40)	
Male	31	17+2 (61.3)	10+2 (38.7)	
Cigarette smoking				0.441
Yes	28	14+2 (57.1)	10+2 (42.9)	
No	8	6 (75)	2 (25)	

a: Positive Num means the number of positive specimens; b: N1 and N2 denote the numbers of samples expressing Twist1 protein either in the nucleus or in the cytoplasm, respectively; B: signifies the number of samples expressing Twist1 protein both in the nucleus and cytoplasm

pression was found in a breast cancer research [12], and difference in Twist1 protein expressions was also explained by post-transcriptional regulation. In contrast, a previous detection consisting of 8 paired BUC tissues and normal mucosas, found that the positive rate of Twist1 mRNA in malignant tissues (100%) was significantly higher than in paired normal mucosas (12.5%), in accordance with its protein expression [11]. Nevertheless, review of the literature revealed consensus that Twist1 protein expression was mainly associated with post-transcriptional regulations (post-translational and translational modification). Many studies found that the phosphorylation degrees of Twist1 protein in tumors positively correlated with its expression levels, suggesting that phosphorylation of Twist1 protein stabilized its expression[13,14,20,21]. On the other hand, a very recent study found several putative regulatory elements at the Twist1 3'UTR (untranslated regions), consisted of miRNA target sites and two cytoplasmic polyadenylation elements, which could restrain the Twist1 protein translation through specifically binding with miR-580 etc [22]. Although these findings could partly explain our results, more studies are needed to clarify the relationship between Twist1 mRNA and protein expression.

In conclusion, our study demonstrated that Twist1 protein expression in BUC specimens was significantly higher than the adjacent non-cancerous mucosas and was not associated with clinicopathological features. Moreover, Twist1 protein was mainly distributed in the nucleus and expressed in the mesenchymal cells of several malignant specimens; Twist1 mRNA expression in malignant specimens were significantly lower than in paired non-cancerous mucosas (discordance with Twist1 protein expression), and correlated with grade and TNM stage.

Finally, we conclude that Twist1 may play an oncogenic role during tumorigenesis and development of BUC, and its abnormal protein expression might

Table 3. Relationship between the expression of Twist1 protein and clinicopathological features

Groups	*N*	-	+	++	+++	%	*p-value*
BUC	40	4	27	7	2	90.0	0.000[a]
Non-cancerous	14	13	1	-	-	7.14	
Tstage							1.000[b]
T_{a-1}	27	3	18	5	1	88.9	
T_{2-4}	13	1	9	2	1	92.3	
Grade							0.673[c]
1	8	1	5	2	-	85.5	
2	26	3	18	4	1	88.5	
3	6	-	4	1	1	100.0	
Age (years)							0.730[d]
≤50	12	2	7	3	-	83.3	
>50	28	2	20	4	2	92.9	
Gender							0.493[e]
Female	6	1	4	1	-	83.3	
Male	34	3	23	6	2	91.2	
Cigarette smoking							0.543[f]
SI≥1	30	2	23	4	1	93.3	
SI<1	10	2	4	3	1	80.0	

a,c:Pearson's x^2test; b,d,f: Correction x^2test; e:Fisher's exact test; SI: smoking index; BUC: bladder urothelial carcinoma

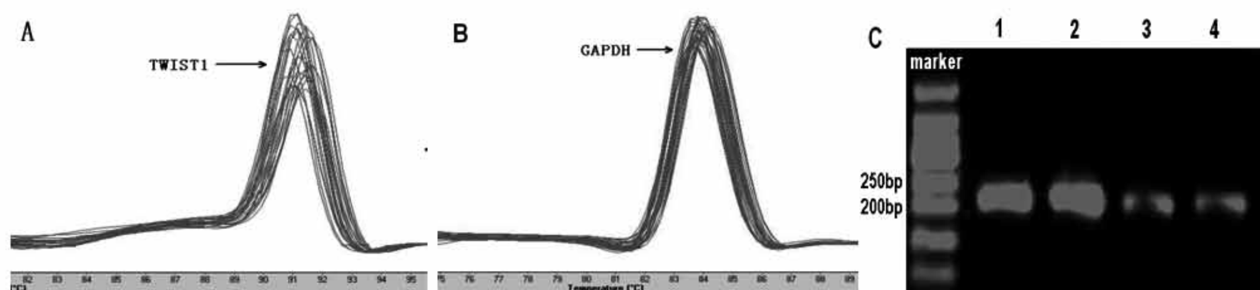

Figure 1. Melting peaks of RFQ-RTPCR products and DNA gel electrophoresis.
A, B: Melting peaks of target gene Twist1 and reference gene GAPDH, respectively;
C: DNA gel electrophoresis of Twist1 (201bp); 1-4 indicate different samples.

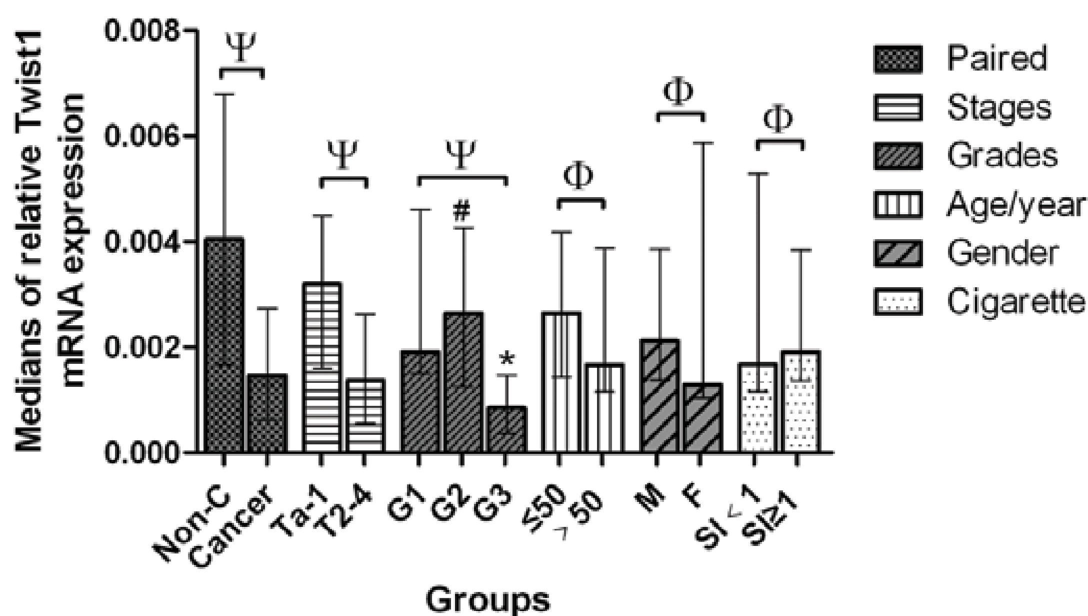

Figure 2. Medians and interquartile ranges of relative Twist1 mRNA expression in different groups.
Non-C: paired non-cancerous mucosa; Ψ: $p<0.05$; Φ: $p>0.05$.
*:G3 vs G1, $p<0.05$; G3 vs G2, $p<0.05$; #: G1 vs G2, $p>0.05$.

be associated with the disordered regulations after transcription. Twist1 may be utilized as a new potential biomarker for BUC. Undoubtedly, in order to further verify the correlations between Twist1 expression and clinicopathological features, multicentric clinical researches and followup studies are required. Meanwhile, more fundamental research are needed to further elucidate the regulatory mechanisms beneath Twist1 expression, as well as Twist1-related signal pathways involved in BUC.

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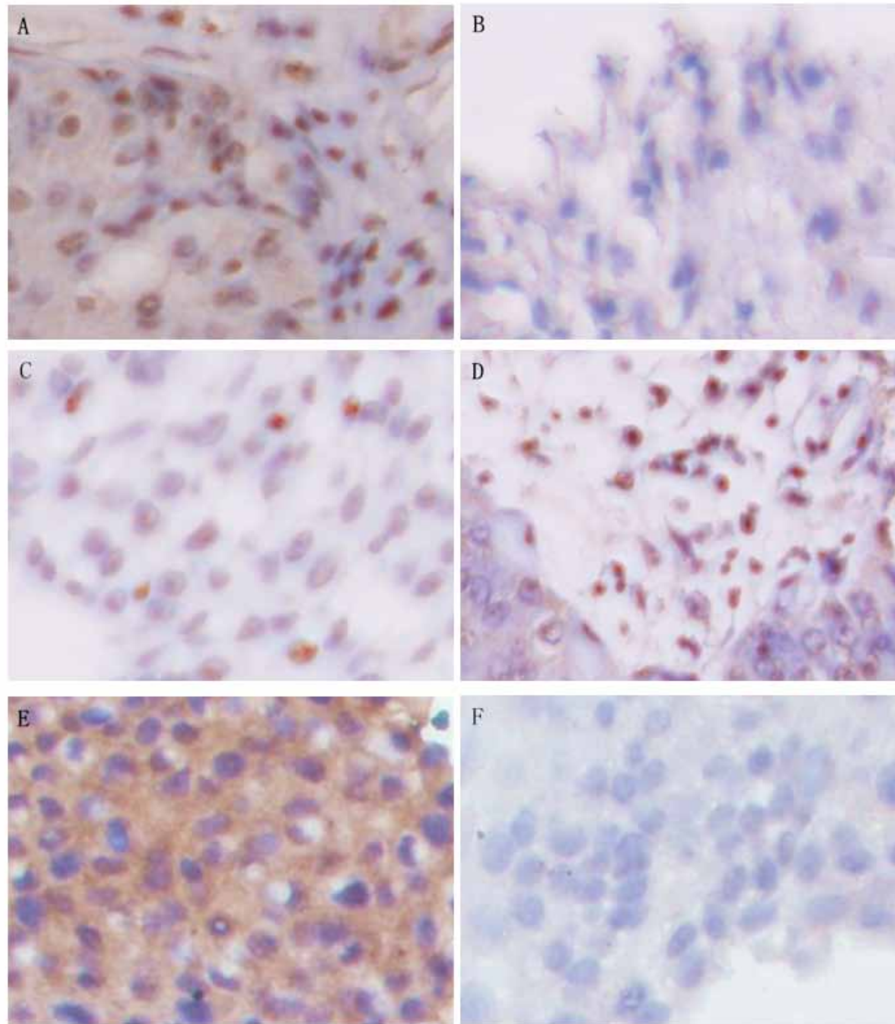

Figure 3. A,B,C,D,E,F: Expression of Twist1 protein in BUC tissues and paired non-cancerous mucosa (EliVision^{TM}, ×400).
A&B: Positive and negative expression of Twist1 protein in a BUC tissue (G2,T1) and paired normal mucous membrane, respectively; C: Twist1 protein expressed in a BUC tissue (G2,T1); D: Strong positive nuclear expression of Twist1 protein in the mesenchymal cells of a BUC tissue (G2,T1); E: Strong positive cytoplasmic expression of Twist1 protein in a BUC tissue (G2,T2b); F: Negative expression of Twist1 protein in a BUC tissue (G2, T2a).

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