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ORIGINAL ARTICLE

The correlation between TWIST, E-cadherin, and beta-catenin in human bladder cancer

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Summary

Purpose: *Epithelial-to-mesenchymal transition (EMT)-related factors are known to contribute to the invasion and migration of multiple cancers. However, the expression levels of and the relationship between TWIST, E-cadherin, and beta-catenin in bladder cancer are not yet known. Therefore, this study investigated the relationship between TWIST, E-cadherin, and beta-catenin in tissue specimens and cell lines of bladder cancer.*

Methods: *Microarrays of bladder cancer tissue and bladder cancer cell lines were used to study the expression levels of TWIST, E-cadherin, and beta-catenin, with disease stage and grade using immunohistochemistry. Moreover, the siRNAs of TWIST, E-cadherin, and beta-catenin were transfected into the bladder cancer cell lines to study any relationship between these factors.*

Results: *The levels of TWIST and beta-catenin were up-regulated with increasing grade of malignancy. In contrast, the corresponding results for E-cadherin were just the opposite. Furthermore, inhibition of the expression of TWIST elevated the expression of E-cadherin, but reduced the expression of beta-catenin. However, reduction of beta-catenin by siRNA had no influence on TWIST, but up-regulated the expression of E-cadherin.*

Conclusion: *TWIST may act upstream of E-cadherin, which can indirectly regulate the expression levels of beta-catenin. The EMT factors TWIST, E-cadherin, and beta-catenin may be a cluster of biomarkers for the metastatic progression of bladder cancer.*

Key words: beta-catenin, bladder cancer, E-cadherin, TWIST

Introduction

Bladder cancer is one of the most common cancers worldwide. Numerous factors, including genetic polymorphisms and genetic and epigenetic alterations, are involved in the tumorigenesis, progression, and metastasis of bladder cancer [1]. Most cases are of transitional histology; 75% of the patients have non-muscle invasive disease, and 20-30% are muscle-invasive cancers [2]. Despite good prognosis for non-muscle invasive patients, recurrence is common, and up to 30% of recurrences progress to a muscle-invasive condition [2]. Approximately 50% of the patients with muscle-invasive cancer, including those who progress to this state from non-muscle invasive disease, already harbor occult distant metastases and display a poor 5-year survival rate [3]. Therefore, defining the role of various genes involved in tumorigenesis and tumor progression is important.

It is well known that survival depends on the grade and stage of bladder cancer [4]. Moreover, research shows that multiple tumor-associated proteins are associated with the grade of malignancy in bladder cancer. Recently, the expressions of TWIST and E-cadherin were reported to be associated with survival of bladder cancer patients [5,6]. TWIST, a basic helix-loop-helix transcription factor, has been shown to play a key role

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in the metastatic progression and advanced tumor stage of several types of human cancers [7-11]. One of the mechanisms reportedly responsible for the positive effect of TWIST on metastasis is its ability to promote EMT, which is a key step during embryonic morphogenesis and was recently implicated in the metastatic process of several malignancies [12-14]. One of the hallmarks of EMT is the loss of E-cadherin, a central component of cell-cell adhesion junctions, the loss of which has been found frequently in metastatic tumors [15]. Several studies reported that TWIST-induced metastasis is associated with down-regulation of E-cadherin [6,13,15-17].

In addition, EMT is characterized by a switch in beta-catenin location and function, changing from acting as an integral junction-complex component in epithelial cells to forming an active bipartite complex with members of the lymphoid-enhancer factor family of DNA-binding proteins, which then translocates into the nucleus and transactivates target genes that include oncogenes and tumor suppressors [18]. Activated beta-catenin signaling is implicated in the genesis of a variety of tumors. In epithelial cells, the association of the cadherin/catenin complex with the cytoskeleton is essential for tight cell-cell interaction. However, down-regulation of cadherins results in the separation of neighboring cells, a phenomenon that is observed during embryonic development at the EMT of forming mesoderm, as well as in tumor cells, allowing their invasion and dissemination throughout the body [19-21]. In addition, E-cadherin reportedly acts as an inhibitor of beta-catenin/T-cell transcription factor (TCF)-mediated transcription by sequestering beta-catenin at the plasma membrane [22]. Loss of E-cadherin is frequent in transitional cell carcinoma (TCC)[23]. Moreover, E-cadherin is demonstrably involved in the inhibition of WNT /beta-catenin signaling between the TCC lines [24]. Up-regulation of E-cadherin can inhibit cell invasion and migration in TCC lines [5].

The relationships between TWIST, E-cadherin, and beta-catenin have not yet been clarified. Therefore, we undertook this study speculating that TWIST, E-cadherin, and beta-catenin may be involved in the tumorigenesis and metastatic progression of TCC.

Methods

Cell culture for 5637, TSGH8301, BFTC905, HT1376, HT1197 and TCCSUP cell lines and human bladder cancer tissue microarray

Human bladder cancer cell lines, including 5637, TSGH8301, BFTC905, HT1376, HT1197 and TCCSUP purchased from American Type Culture Collection (ATCC), were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, penicillin (200 U/ml) and streptomycin (100 ug/ml) at 37° C in 5% CO_2.

Bladder cancer tissue microarray slides (BL1002), purchased from U.S. Biomax (Rockville, MD) contained 40 cases of human bladder carcinoma and 10 cases of normal tissues with duplicate cores. The pathologic diagnosis of each sample was determined from the manufacturer's data sheet (http://www.biomax.us/tissue-arrays/Bladder/BL1002).

Western blotting

Cells were lysed in M-PER lysis buffer (Thermo Scientific, Rockford, USA) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and the total lysate was separated by SDS-PAGE and immunoblotted with TWIST (Santa Cruz Bio. Inc., Santa Cruz, CA), E-cadherin (BD Biosciences, Mississauga, ON, Canada), beta-catenin (BD Biosciences, Mississauga, ON, Canada), beta-actin (Santa Cruz Bio. Inc., Santa Cruz, CA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Bio. Inc., Santa Cruz, CA) antibodies. Rabbit antiserum (Santa Cruz Bio. Inc., Santa Cruz, CA) was used as secondary antibody for detection of TWIST, and mouse antiserum (Santa Cruz Bio. Inc., Santa Cruz, CA) was used for the detection of E-cadherin, beta-catenin and GAPDH. The expression of GAPDH was used as internal control.

Immunohistochemistry for TWIST, E-cadherin and beta-catenin

The immunohistochemistry was performed as previously described [25]. The tissue microarray slides were stained with primary antibodies (anti-TWIST antibody, anti-E-cadherin antibody and anti-beta-catenin antibody) and further counterstained with hematoxylin. All immunohistochemical steps were carried out according to the manufacturer's instructions by using a LSAB kit (Dako, Glostrup, Denmark). To confirm the expression of TWIST, E-cadherin, and beta-catenin *in vivo*, human bladder cancer (80 samples in 40 cases) and normal (20 samples in 10 cases) tissue specimen microarrays (purchased from GenDiscovery Biotechnology, Inc., USA) were used, comprising 20 samples of normal, 22 of stage 1, 34 of stage 2, 22 of stage 3, and 1 of stage 4 tumors. Immunohistochemistry was used to determine the expressions of TWIST, E-cadherin, and beta-catenin (Figure 1).

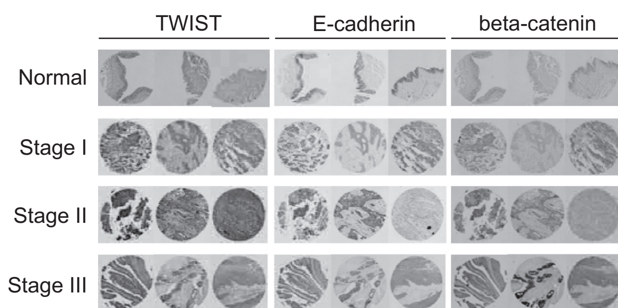

Figure 1. Detection of the expression of TWIST, E-cadherin, and beta-catenin in human bladder specimens. The human bladder cancer tissue microarray was used to determine the expression of TWIST, E-cadherin, and beta-catenin by immunostaining including 20 samples of normal, 22 of stage 1, 34 of stage 2, and 22 of stage 3 disease; 3 of the specimens are shown.

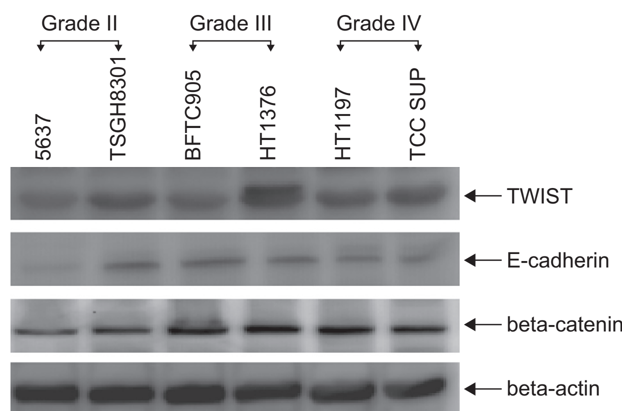

Figure 2. TWIST, E-cadherin, and beta-catenin expressions in multiple human bladder-cancer cell lines. The total protein collected from various human bladder-cancer cell lines were determined with Western blotting using anti-TWIST, -E-cadherin, -beta-catenin, and -beta-actin antibodies. The beta-actin was used as an internal control.

siRNAs and transfection

The siRNA plasmid construction of TWIST, E-cadherin and beta-catenin were obtained from the National RNAi Core Facility, Taiwan. The siRNA was transfected into human bladder cell lines (5637 cells) with lipofectamine 2000 (Invitrogen, CA, USA), and the expression levels of the proteins were detected by Western blotting after 48 h post-transfection.

Statistical analysis

The results were expressed as means ± standard error of mean (SEM) for each experiment. Comparisons between groups were performed by Student's *t*-test. P-values less than 0.05 were considered statistically significant.

Results

The expression levels of TWIST, E-cadherin, and beta-catenin in human bladder cancer cell lines

Human bladder cell lines were divided into 3 groups by tumor grade with the original operative specimen described in the ATCC illustration. The expression levels of TWIST, E-cadherin, and beta-catenin were determined by Western blotting. Both TWIST and beta-catenin had a tendency to increase in a grade-dependent manner (Figure 2). On the contrary, the expression of E-cadherin decreased in a grade-dependent manner (Figure 2).

The expression levels of TWIST, E-cadherin and beta-catenin in vivo with human bladder tissue microarray

Because cultured cell lines often have different characteristics compared with the original tumor the expression profiles of TWIST, E-cadherin, and beta-catenin showed a tendency to increase or decrease in a grade-dependent manner (Figure 2), but with lack of integrity.

The expression of TWIST and beta-catenin showed a tendency to increase in a stage-dependent manner (especially in stage 1 and 2; $p < 0.001$ for normal tissue vs. stages 1, 2, and 3) in human bladder specimens (Figure 3). In contrast, the expression of E-cadherin had a tendency to decrease in a stage-dependent manner (Figure 3; $p < 0.001$ for normal tissue vs. stage 3). However, the expression of these factors showed no correlation with the grade (data not shown).

The relationship between TWIST, E-cadherin and beta-catenin in human bladder cancer cell lines

TWIST and beta-catenin expressions in human bladder specimens and cancer cell lines showed a trend for elevation (Figures 2 and 3). To reveal the relationship between TWIST, E-cadherin, and beta-catenin, siRNA of TWIST and beta-catenin knockdown was used. The expressions of TWIST and beta-catenin in TWIST, E-cadherin, and beta-catenin siRNA-knockdown cells were confirmed by Western blotting (Figure 4). All the expressions of TWIST, E-cadherin, and beta-catenin were determined, and we found that knocked-down TWIST could up-regulate the expression of E-cadherin, but suppress the expression of beta-catenin (Figure 4). Knockdown of the expression of E-cadherin could elevate the expression of beta-catenin but had no influence on TWIST. In addition, decrease of the beta-catenin in siRNA-knockdown cells could not influence

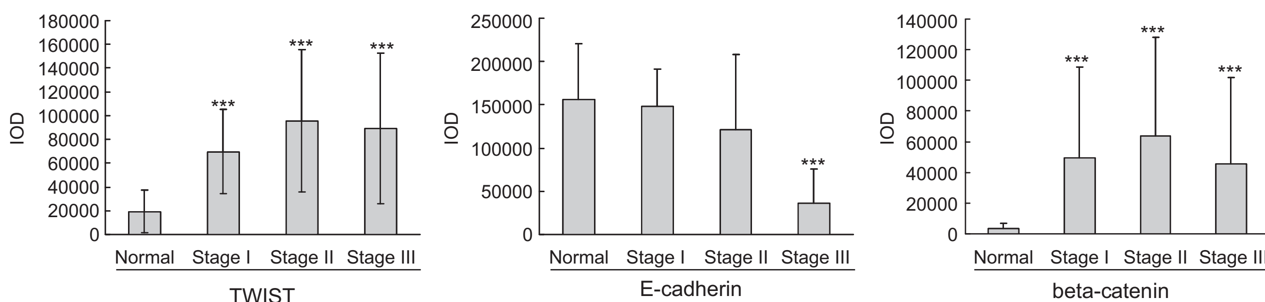

Figure 3. The expression level of TWIST, E-cadherin, and beta-catenin was quantified using ImageJ software and organized by clinical stage. All of the digital images of each specimen were captured and measured as the integrated optical density (IOD), equal to the average density x area. **p<0.01 ***p < 0.001.

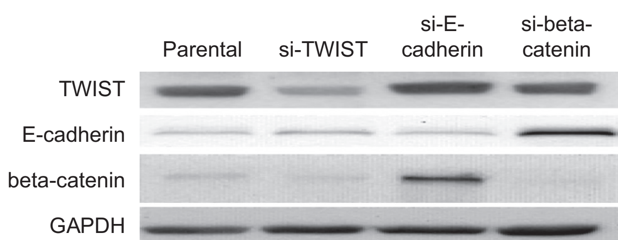

Figure 4. The relationship between TWIST, E-cadherin, and beta-catenin in human bladder-cancer cells. siRNA of TWIST, E-cadherin, and beta-catenin was transfected individually into 5,637 human bladder cancer cell lines. The expression and the relationship between TWIST, E-cadherin, and beta-catenin were evaluated by Western blotting. GAPDH was used as an internal control.

the expression of TWIST. The expression of E-cadherin was up-regulated in the beta-catenin-knockdown cells (Figure 4).

Discussion

This study demonstrated that the expressions of TWIST and beta-catenin have a positive correlation/tendency with the stage progression of human bladder cancer. However, the expression of E-cadherin has a negative correlation/tendency with the stage progression of bladder cancer. Moreover, we demonstrated that TWIST is the up-stream of E-cadherin and could elevate beta-catenin expression through reduction of E-cadherin. Therefore, TWIST, E-cadherin, and beta-catenin may be used as pathologic markers for a stage-progression prediction. However, more analyses are required to support/confirm these findings with a larger cohort of studies in bladder cancer or other cancers.

Several studies have revealed that TWIST-induced metastasis is associated with down-regulation of E-cadherin, indicating its involvement in TWIST-mediated metastasis in multiple human cancers [6,13,15-17]. In addition, E-cadherin reportedly acts as an inhibitor of beta-catenin/TCF-mediated transcription by sequestering beta-catenin at the plasma membrane [22,26]. E-cadherin is also involved in the inhibition of WNT/beta-catenin signaling in bladder cancer cell lines [24]. Up-regulation and/or activation of beta-catenin signaling have been implicated in the progression of a variety of tumors [22]. The findings of our study are consistent with previous reports that the expression levels correlated with tumor stage with an elevation in TWIST and beta-catenin and a decrease in E-cadherin expression [6,13,15-17]. Most importantly, we provided a global view of the expressions of TWIST, E-cadherin, and beta-catenin in human bladder cancer.

Furthermore, the relationship between TWIST and E-cadherin and E-cadherin and beta-catenin, separately has been reported by several authors [6,26,27]. However, to date, no study has reported the relationship between TWIST, E-cadherin, and beta-catenin. In the present study, we demonstrated that TWIST acts up-stream of E-cadherin and beta-catenin. Moreover, down-regulation of E-cadherin could up-regulate the expression of beta-catenin and has no influence on TWIST. Similarly, knockdown of beta-catenin with siRNA showed no influence on the expression of TWIST. Knockdown of beta-catenin with siRNA could up-regulate the expression of E-cadherin in human bladder cancer cells. However, the E-cadherin promoter can bind to the beta-catenin/lymphoid enhancer factor (LEF)-1 complex to regulate the transcription of E-cadherin. Therefore, the E-cadherin itself is reportedly a target of beta-catenin [28], and this finding was identical to our results. In brief, TWIST, E-cadherin, and beta-catenin may represent a cascade, and TWIST can up-regulate the expression of beta-catenin to promote highly aggressive cancers through E-cadherin suppression in human bladder cancer. Our findings may provide a new strategy for the study of anti-invasive cancer targeting therapies.

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