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Upregulation of Twist2 in Non-Muscle Invasive Urothelial Carcinoma of the Bladder Correlate with Response to Treatment and Progression

Mohamed Wishahi[1*], Heba Khalil[2], Mohamed H. Badawy[1], Amr Elkholy[1], Khaled Eseily[1], Shady Anis[3], Samir Eldahshan[1], Noura Kamel[4], Mahmoud Romeih[5]

[1]Urology Department, Theodor Bilharz Research Institute, Cairo, Egypt; [2]Pathology Department, Theodor Bilharz Research Institute, Cairo, Egypt; [3]Pathology Department, Faculty of Medicine, Cairo University, Cairo, Egypt; [4]Pathology Departments, National Research Centre, Cairo, Egypt; [5]Biochemistry Department, Theodor Bilharz Research Institute, Cairo, Egypt

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***Correspondence:** Mohamed Wishahi. Urology Department, Theodor Bilharz Research Institute, Cairo, Egypt. E-mail: wishahi@gmx.net

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Abstract

BACKGROUND: Twist2 is a transcription factor and an epithelial-to-mesenchymal transition that plays an important role in cell polarity, cell adhesion, and has a role in tumour invasion and metastases.

AIM: In this study, we examined the expression of Twist2 in non-muscle invasive bladder carcinoma (NMIBC) and correlated the expression with response to treatment and tumour progression.

METHODS: Data of 305 patients with NMIBC of Ta, T1 were retrieved from hospitals archives. Twist2 expression was examined in tissue samples by immunohistochemistry at initial diagnosis and final follow-up, normal control was 10 normal urothelium, 10 patients with muscle-invasive bladder cancer (MIBC) were a positive control. Treatment of NMIBC was implemented according to the European Association of Urology guidelines on NMIBC. The descriptive statistical analysis included means, standard deviation, p-value; Univariate and multivariate Cox regression analyses.

RESULTS: Twist2 expression score was identified as negative, low (1-15%); medium (15-40%); and high (40-100%). Patients who had low or low medium scores at the initial diagnosis had a good response and a favourable prognosis. Expression of a high score of Twist2 in patients having high-grade T1 tumours showed non-responsiveness to repeated courses of intravesical bacillus Calmette Guerin (BCG) therapy and was upstaged to MIBC.

CONCLUSION: Twist2 expression in tissue samples of NMIBC would indicate the tumour response to therapy, upgrading and upstaging in the follow up after intravesical BCG therapy.

Introduction

Bladder cancer (BCa) is seventh cancer in the male population, 75% of patients with BCa present at initial diagnosis with non-muscle invasive bladder cancer (NMIBC) of stage Ta and T1 respectively [1]. Muscle invasive bladder cancer (MIBC) of stages T2-T4 has the potential of lymph nodes invasion and distant metastases. High-grade Ta and T1 tumours have a high potential for recurrence and upstaging to MIBC. The twist is a basic helix-loop-helix transcription factor, two Twist-like proteins, Twist1 and Twist2, are sharing structural details, the N-termini of both Twists is different where Twist1 lacks glycine-rich region that consequently leads to a different function of Twist2 [2]. The epithelial-mesenchymal transition (EMT) leads to the loss of cell polarity and cellular adhesion that facilitates cancer cells migration, invasion, and metastases in different types of cancer including BCa. Twist2 is a regulator of EMT that has an important role in tumorigenesis where malignant cells acquire the ability of invasion, metastasis in addition to resisting cancer therapy [3] [4] [5]. In breast cancer, Twist2 expression was significantly increased, the cytoplasmic Twist2 in cancer cells at the tumour centre of primary carcinomas contributes to the maintenance of epithelial cancer characteristics,

invasion and metastasis. Heterogeneous expression of Twist2 in tumours may have a functional link to tumour progression, Twist2 promotes breast cancer invasion through loss of E-cadherin, and there is a strong link between nuclear Twist2 represented as nuclear positivity with an overexpression score and cancer progression and metastases [6] [7]. EMT process depends on Twist2 cellular location [5]. EMT is a dynamic process in the pathogenesis of BCa, despite significant advancements in diagnosis and treatment, the outcomes remained more or less the same. Expression of EMT markers E-cadherin, N-cadherin, Vimentin, Snail, Twist, Zeb, and Slug in BCa showed that increased expression of EMT transcription factors correlated significantly with tumour grade and stage; therefore EMT marker profile would provide a sensitive and effective prognostic tool for investigation of BCa [8]. Expression levels of EMT markers Twist in tumour specimens taken by transurethral resection of bladder tumour of patients with NMICB and measured by immunohistochemistry(IHC) staining showed that expression level was significant predictors of intravesical recurrence-free survival, the nuclear positivity of Twist2 related to recurrence and progression [8]. In a Long-term follow-up of the patient to determine whether EMT related markers can predict patient survival and progression in NMIBC, it was shown that EMT markers E-cadherin, Twist, and Vimentin detected by IHC had statistically significant correlations with grade, recurrence, tumour progression, and progression-free survival [9]. The twist is considered as a potential oncogene promoting the proliferation and inhibiting the apoptosis in BCa; it promotes the synthesis of a pro-angiogenic factor, a vascular endothelial growth factor which is involved in tumour progression and metastasis [10]. Upregulation of Twist2 was related to the aberrant expression of E-cadherin and the increased expression of Vimentin, which were reported as important indicators of EMT. Twist2 regulates EMT by depriving the epithelial cell phenotype of E-cadherin and endowing the mesenchymal cell phenotype with Vimentin, which may be involved in the progression and prognosis [11]. Tumour progression in colorectal cancer was significantly correlated to overexpression of Twist2 and Twist1 [12]. Twist2 expression was correlated to the progression of cervical cancer [13]. The purpose of this study was to determine whether Twist 2 upregulation was the promoter of NMIBC not to respond to intravesical bacillus Calmette Guerin therapy (BCG) and whether the overexpression was correlated positively with recurrence and upstaging. The goal of this study was to evaluate Twist2 expression by IHC in order to track disease response and progression during therapy, as well as the evaluation of association between Twist2 value and the clinical outcome, and/or determination of whether Twist2 expression contributes to providing additional information about the likely clinical outcome beyond the information provided by clinicopathological data.

Material and Methods

A cohort of the study was 305 patients with NMIBC whom tumour samples were stored in tumour archives; the study ran between the years 2008 and 2016. Patient's clinical data were analysed, and the selection was made for those who had at least 3 years of potential follow-up or had frequent recurrences, upgrading or upstaging to MIBC that indicated immediate cystectomy, inclusion criteria were no metastatic disease at diagnosis, any previous chemotherapy or radiotherapy. Ten patients with MIBC tumours were considered as positive control, 10 patients who had benign prostatic hyperplasia and undergone trans-urethral resection of the prostate (TURP) and had no associated cancer were taken as negative control.

The institutional review board approved the study and patients gave informed consent to use the medical data; the study was to evaluate tumour markers in diagnosis and follow-up for NMIBC.

Patients were adults aged from 32 to 83 years, diagnosis of NMIBC was based on urine cytology, abdominal ultrasound, non-contrast computed tomography of the urinary tract, cystoscopy and transurethral resection of bladder tumours (TURB), pathological diagnosis of NMIBC was made by two independent pathologists. Patients received treatment in accordance with the European Association of Urology guidelines for the treatment of NMIBC [14], patients who had low papillary grade Ta tumors were treated with TURB, recurrent low-grade Ta and low-grade T1 tumors were treated with complete TURB and 1 year of full-dose BCG treatment, patients with recurrent tumours with high grade and high-risk tumours, a full-dose of intravesical BCG for 1–3 years were indicated. Tumour progression to a higher grade or upstaging an immediate cystectomy was done. Follow up was done with urine cytology and cystoscopy and biopsy every 3 months for 36 months, patients who had a recurrence after the first course underwent a re-TURB and second course of intravesical BCG. Patients showed repeated recurrence, upgrading, or upstaging to muscle-invasive bladder cancer categorised as non-responder to BCG intravesical therapy.

Patients characteristics

A group of 305 patients with NMIBC who had been treated with either TURB alone for low papillary grade Ta a tumour, or TURB and Intravesical intravesical BCG for patients with high-grade Ta, and for T1 tumours, follow-up took place for 3 years. Patients were categorized into 4 groups; low grade Ta (n = 32) were group 1, high grade Ta (n = 67) were group 2, low grade T1 (n = 47) were group 3, high grade T1 (n = 114) were group 4 (Table 1).

Table 1: Demographic and Clinical Characteristics of patients having Ta-T1 non-muscle invasive bladder carcinoma at baseline and at follow-up following treatment with TURB* and BCG‡

Features	Ta, Low grade	Ta,High grade	T1,low grade	T1, high grade
Number	32	67	92	114
Gender				
Male	27	50	69	78
Female	5	17	23	36
Age-years				
Range-mean	32-76 (51)	48-76 (62)	47-82 (63)	45-83 (65)
Treatment	TURB*	TURB + BCG‡	TURB + BCG	TURB + BCG
Response to treatment	Cured	Responder (68.6%)	Responder (54.3%)	Recurrence (42.4%)
		No response (31.3%)	No response (45.6%)	Upstaging (67.5)
Follow-up Diagnosis	Cured	Ta, low grade, n = 46 T1, high grade, n = 21	T1, low grade, n = 50 T1, high grade, n = 30 T2-T4, n = 12	Recurrent T1, n = 37 T1-T3, n = 77

*TURB, transurethral resection of a bladder tumour; ‡ BCG, intravesical instillation of bacillus Calmette Guerin.

Response to treatment was either good response with no recurrence or residual diseases in the 3 years follow-up, or non-responder with repeated recurrences, upgrading, or upstaging. According to response criteria, the 305 patients were categorised into 6 groups in consideration of initial diagnosis and endpoint of being responder or non-responder. All patient's tissue samples at initial diagnosis and at last follow-up were studied for Twist 2 expression by immunohistochemistry, the follow-up groups were: group1, low grade Ta responder (n = 32); group2, High grade Ta non-responder (n = 46); group3, high grade Ta non-responder (n = 21); group 4,low grade T1 responder (n = 50),group 5, Low grade Ta non-responder (n = 42), group 6, high grade T1 non-responder (n = 114) (Table 2).

Table 2: Twist2 expression in non-muscle invasive bladder cancer at baseline in the intention-to-treat and at follow up with correlation to response to BCG therapy*

Group characteristics	no	Twist 2 expression at initial diagnosis Range	Mean ± SD¶	Score†	Twist2 expression at final follow-up Range	Mean ± SD	Score	p[9] value
1.Ta low grade responder ‡	32	0	0	Negative	0	0	Negative	0.001
2.Ta high grade responder	46	3-13	1 ± 1	Low	3-6	3 ± 1	low	0.001
3.Ta high grade nonresponder‡	21	21-30	26 ± 2	Medium	27-38	35 ± 1	Medium	0.001
4. T1 low grade responder	50	25-36	27 ± 3	Medium	20-25	23 ± 1	Medium	0.001
5.T1 low grade nonresponder	42	45-55	50 ± 1	High	60-75	65 ± 1	High	0.001
6. T1 high grade nonresponder	114	57-83	65 ± 3	High	74-92	88 ± 3	High	0.001

*Expression of Twist2 by immunohistochemistry was evaluated at initial diagnosis and final follow-up of Ta-T1 non-muscle invasive bladder cancer, patients were treated with Trans-urethral resection and intravesical BCG for 3 courses, at the end of follow-up patients were stratifies into 6 groups according to response to treatment. ‡ Response was assessed at the final follow-up, responder was identified as complete response after treatment, Responders are patients who had complete response with no or minimal residual disease, nonresponders are patients who received maximum of three courses of transurethral resection of a tumour and three courses of intravesical instillation of BCG, this patient had upgraded, upstaging, and or repeated recurrences. †Twist2 expression score was measured according to the percentage of cytoplasmic and nuclear staining: negative staining, low score 1-15%, medium 15-40%, high 40-100 % positive. The P value was calculated with the use of chi-square test, P= 0.001 was statistically significant. ¶ SD standard deviation. BCG; Bacillus Chalmette Guerin intravesical installation.

Immunohistochemistry

Total number of cases was of 305 patients; tissue samples that were taken by TUR-BT in the initial diagnosis and in the follow up and were examined for expression of Twist2 by immunohistochemistry (IHC), Normal control were tissue samples of patients having normal urothelial tissue and had not bladder cancer or other malignancies (*n* =10), positive control were muscle invasive urothelial bladder cancer who underwent cystectomy (*n* = 10) (Table 3).

Table 3: Clinical features and Twist2 expression in the negative and positive control groups

Group	Number	Age Range (mean)	Gender Male	Female	Twist2 expression Range	Mean ± SD	Score
Normal urothelium	10	58-76 (67)	10	0	0	0	Negative
Muscle invasive T2	10	57-76 (62)	8	2	86-98	95±4	High

SD: standard deviation.

Tissue samples at the initial diagnosis and in the following periods were fixed for 24 hours in 10% neutral buffered formalin solution and then processed for preparing paraffin blocks. Tissue samples were examined for histopathological evaluation with Hematoxylin and eosin stain and evaluated according to the international histological classification of urinary bladder tumours of WHO-1999, tumour grade according to WHO grading system, 2004**,** and tumour stage according to TNM staging system of UICC-2004 2015.

Paraffin-embedded sections of 4mm thickness were processed using Anti-Twist2 concentrated antibody (Abcam, ab 57997, USA), antigen retrieval was performed in all cases by steam-heating the slides in 1 mmol/l solution (pH 9.0) for 45 min. After blocking of endogenous biotin, staining was performed using an automated immunostainer (Ultra Benchmark, Ventana, USA), followed by detection using Ultraview detection Kid (Ventana, USA). Positive and negative control sections were used for each assay.

Immunohistochemistry scoring

The entire section was examined to find the area with maximum positivity, and stained nuclei for Twist2, positively stained cytoplasm and nuclei were scored using the 40X objective in 20 fields. Immunohistochemistry evaluation was done by two independent observers. For evaluation of a Twist2 expression, each slide was scored according to the percentage of positively stained cytoplasm and nuclear staining. The following ranges were used: negative score was that to cells not stained, low score was that 1 to 15% positive cytoplasmic stain, medium score was to 15-40% stain, high expression score was to over 40% cytoplasmic expression associated with nuclear expression, normal urothelium was negative

for Twist2 expression, muscle-invasive urothelial carcinoma showed high expression of Twist2 (Figure 1). Negative external control was done by omitting primary antibodies. The acquisition of images was done with Nikon Eclipse E600 software program Lucia 5.

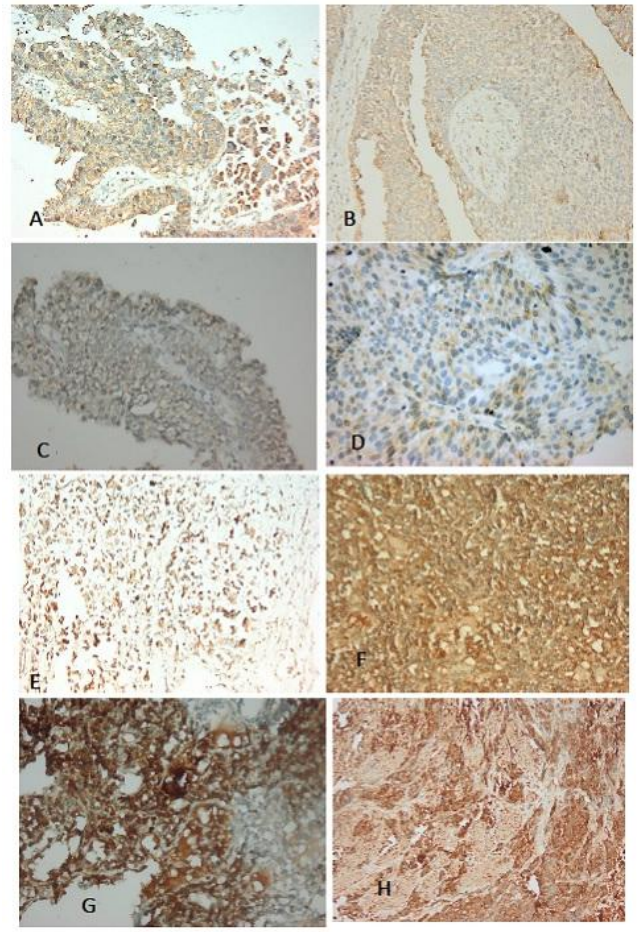

Figure 1: Immunohistochemical (IHC) staining of Twist2 expression on paraffin-embedded tissues of non-muscle invasive bladder carcinoma. No expression of Twist2 could be seen in normal urothelial tissues, while positive expressions of Twist2 have mainly localised in the cytoplasm in high-grade Ta, and in both cytoplasm an nucleus of high-grade T1 and T2. Magnitude ×200, ×400. (a) High-grade Ta, positive for Twist2, with medium expression, IHC x200; (b) Low-grade Ta, positive for Twist2, with low expression, IHC, x200; (c) High-grade Ta, positive for Twist2, with high expression, IHC x200; (d) Low grade papillary Ta, positive for Twist2, with low expression, IHC x 400; (e) High grade T1, positive nuclear and cytoplasmic staining with overexpression, IHC x200; (f) High grade T1, Positive for Twist2,with overexpression, IHC x400; (g) High grade T1, Positive for Twist2,with overexpression, IHC x200; (h) muscle invasive carcinoma T2, highly positive for twist2, with overexpression, IHC x200

Statistical analysis

A descriptive study of variables in the study cohort was done; Chi-square test was performed to compare categorical variables with the expression of Twist2, dependent variables were recurrence and progression-free survival. Differences between categories were evaluated using a log-rank test. To determine the way by which a tumour was not responding to BCG intravesical immunotherapy, recurrence, and how tumour progression was affected by upregulation of Twist2 expression, a Cox's proportional hazards analysis was performed. A $p < 0.05$ was accepted as statistically significant. Unavailable Cox regression analysis was performed with theTwist2 marker treated as a continuous variable, as proposed by Almman et al., [15] and Keegan et al., [16], trying to avoid extreme bias. The expression of Twist2 variables was analysed in function of recurrence-free and progression-free survival in response to treatment.

Results

The 305 patients with NMIBC were 224 men and 81 women, with age ranging from 32 to 83 years; patients with Ta tumors were 99 cases, 32 with low-grade papillary tumor, and 67 with high grades Ta; patients with T1 amounted to 206 cases, 92 of low grade, and 104 were high grade (Table1). The expression of the Twist2 in the tissue samples was measured in the initial pathological diagnosis as well as in the follow-up, Patients were categorised according to initial diagnosis and final diagnosis in the follow up after therapy with TURB alone or TURB and intravesical BCG for 2-3 courses. Clinical data reported cure, recurrence, upgrading, and upstaging, during the 3 follow-up years, tumour responded to therapy was considered responder, non-responders were the tumours that showed upgrading and upstaging (Table 2). IHC for expression of Twist2 was done in the initial diagnosis, and in the final follow-up, patients were categorised into six groups (Table 3). Interpretation of the Twist2 expression showed that the higher is the stage and grade of a tumour, the higher the Twist2 expression scores. Patients who did not respond to treatment and had upgrading and upstaging showed higher expression of Twist2 in the initial diagnosis the more likely the poor response therapy, these findings were observed in groups 3,5, and 6 who were high-grade Ta, part of low-grade T1, and high-grade T1. High score at initial diagnosis in patients with high-grade T1 was correlated with non-responsiveness to therapy and upstaging to MIBC. Low-grade Ta papillary tumours had a low score in the initial and follow-up diagnosis and had a good prognosis.

High grade Ta tumor were two subdivisions, those with low medium score in the initial diagnosis (n = 47) who had responded to therapy and had the same low medium score in the follow up, the second subdivision were those who had high medium score in the initial diagnosis (n = 33), they were upstaged to high-grade T1 in the follow-up. Comparison between mean values of Twist2 expression in initial diagnosis of NMIBC and MIBC was highly significant (0.001),

comparison between mean values of Twist2 expression in initial diagnosis of NMIBC and non-responders that had upstaged and upgraded in the follow-up was highly significant (0.001) (Table 2) Univariable analysis of Twist2 expression in the initial diagnosis in relation to tumor grade and stage was highly significant (P 0.001); univariable analysis of Twist2 expression in relation to sex and gender was insignificant. Univariate logistic regression model showing Twist2 Expression measured at initial diagnosis is a predictor of disease prognosis (P-value 0.002), Adjusted R Square (0.571), odds ratio (1.416) (Table 4)

Table 4: Univariate logistic regression model showing Twist2 measured at initial diagnosis as a predictor of disease prognosis

	Adjusted R Square	B	P value	Odds ratio	95% CI
Twist2 expression at initial diagnosis	0.571	0.348	0.002	1.416	1.138-1.761

Discussion

Epithelial-to-mesenchymal transition (EMT) is characterised by loss of cellular adhesion and polarity and is responsible for cancer metastasis and upstaging, EMT regulates urothelial tumour progression and sensitivity to drug therapy [16]. The present study showed that the Twist2 which is one of EMT transcriptional repressors of E-cadherin when measured at initial diagnosis of NMIBC would indicate a response to drug therapy, progression, upgrading and upstaging. Twist2 was reported to be good prognostic markers for tumour progression in urothelial bladder cancer [6] [7] [8] [9] [16]. The present study explored the significance of measurement of Twist2 expression by IHC at initial diagnosis of NMIBC and at follow up following treatment with TURT and intravesical BCG therapy, our finding showed that Twist2 expression score is a predictive marker for tumor response to therapy and would indicate future favorable response or progression, present work is the first study to show the significance of Twist2 expression in NMIBC to predict response to treatment. Overexpression and high medium Twist2 expression score was a determinant factor for non-response to therapy, upgrading and upstaging; overexpression of Twist2 with over 40% score was significant with a p-value ($p < 0.001$). Previous reports show that heterogeneous nuclear expression of Twist2 in cancer tissue will indicate upstaging, progression, metastases, and poor survival [2] [3] [4] [5] [6] [10] [11] [12] [13]. Present results on the cytoplasmic and nuclear expression of Twist2 confirmed the previous reports. There was a significant difference in the expression of Twist2 in NMIUBC and positive control of MIUBC. Using the ROC curve for the diagnostic index of Twist2 with a cut-off > 40, area under the ROC curve was 0.994 with a sensitivity of 100 and specificity of 97.5. The positive predictive value was 87, and the negative predictive value was 100. The percentage of cases that had upstaged or upgraded in the follow-up assessment amounted to 62.95%. The univariate logistic regression model showing Twist2 expression measured at first as a predictor of disease progression showed that the adjusted R square was 0.571, the p-value was 0.002, the Odds ratio was 1.416 with 95% confidence interval of 1.138-1.761. Present results are by previous studies that showed the high positive expression of Twist2 indicating progression, metastases and poor survival in carcinoma of the bladder and other cancers as breast cancer, colorectal cancer, and ovarian cancer [5] [9] [10] [11] [12].

In conclusion, the high expression of Twist2 with intense cytoplasmic and nuclear staining with duplication or triplication of its value in the follow-up for patients with NMIUBC indicated recurrence, upstaging and progression of Ta and T1 tumours and unfavourable response to intravesical instillation of BCG. The study concluded that Twist2 expression in the initial diagnosis and the follow-up of treatment of NMIBC might be useful for stratifying patients with Ta and T1 into risk categories. The results may have an important clinical implication. Twist2 as a marker for EMT would be considered as a tumor marker to predict bladder cancer pathway.

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