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Transforming Growth Factor Beta1 Expression in Cancer-Associated Fibroblasts of Urinary Bladder Cancer: Crucial Applications and Deep Insights

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Abstract

BACKGROUND: Urinary bladder carcinoma (UBC) is one of the most common malignancies in Egypt and all over the world. Transforming growth factor beta (TGFB) levels in plasma and urine were proved to connote predictive and prognostic attributes in UBC patients. Furthermore, Cancer-associated fibroblasts (CAFs) are, now, recognized as a key player in carcinogenesis. Yet, TGFB1 expression in CAFs of UBC had not been elucidated. Moreover, TGFB1-targeted therapy is now emerging with potential benefits for TGFB1 expressing cancers.

AIM: We dedicated this study to explore potential implications of TGFB1 immunohistochemical expression in CAFs of UBC by correlating it to relevant clinical and pathological data.

MATERIALS AND METHODS: This retrospective study included 48 UBC specimens. Different tumor grades were presented in balanced groups. TGFB1 immunohistochemical expression was evaluated, categorized as low or high and compared in CAFs among different UBC grades, statistical analysis of the results was then followed.

RESULTS: TGFB1 expression in CAFs was significantly different among tumor histologic types ($p = 0.01$), high tumor grade ($p \leq 0.01$), presence of muscle invasion ($p \leq 0.001$), higher tumor stage ($p = 0.01$), presence of preceding bilharziasis ($p = 0.003$), and necrosis ($p = 0.03$). There was a highly significant difference between TGFB1 expression in both tumor cells and CAFs ($p = 0.002$). Intense CAFs TGFB1 staining was also strikingly observed along the muscle invading frontside of UBC cells further emphasizing the pivotal role of CAFs expressing TGFB1 in invasion.

CONCLUSION: This study demonstrates significant predictive implications of TGFB1 in UBC, thus emphasizing its potential benefits in management and therapy.

Introduction

Urinary bladder carcinoma (UBC) is the 10th most common form of cancer worldwide with an estimated 573,000 new cases and 213,000 deaths in both sexes in 2020 according to Global Cancer Observatory (GCO). In Egypt, according to GCO, UBC ranking the 3rd most common cancer after liver and prostatic cancers in both sexes of all ages in 2020. The number of new cases in Egypt in 2020 in both sexes and all ages is 10,655/134,632 representing 7.9% of all cancers [1].

More than >95% of bladder tumors are of epithelial origin. The urothelial neoplasms are the most common type followed by squamous and glandular neoplasms [2]. Other less common invasive urothelial cancer types include nested urothelial carcinoma, microcystic urothelial carcinoma, micropapillary urothelial carcinoma, lymphoepithelioma-like urothelial carcinoma, pasmacytoid, signet ring, and diffuse urothelial carcinoma.

The ominous prognosis of UBC is likely attributed to its unpredictable course, histopathological categories of same morphology do not essentially follow the same behavioral course; non-muscle invasion bladder cancer might twist their course to fatal aptitudes; and in contrast, muscle invasive bladder cancer (MIBC) do not necessarily behave more dismally [2].

This enigmatic paradox had inspired researchers to dedicate their studies to highlight potential inputs that might skew the expected course of apparently similar categories as per the traditional prognostication parameters [3].

Among these endeavors, many had emphasized a crucial role of tumor microenvironment (TME) in manipulating the fate of cancer cells [4]. Cancer-associated fibroblasts (CAFs) were one of the prominent contributors proven by many to exert pivotal effect through many attributes involving epithelial mesenchymal transition as well as other influences promoting cancer cells progression and invasion. Transforming growth factor beta1 (TGFB1) was suggested as a labeling marker for CAFs, along with its

additional involvement in cross-talks with cancer cells [5]. Thus, studying TGFB1 in UBC can impart paramount insights in unraveling the underlying mechanisms that could contribute to the unexpected outcomes of morphologically alike UBC cases. Moreover, these insights would not only propose valuable predictive/ prognostic implications but can also endow promising novel TGFB1-targeted therapeutic options [6].

Materials and Methods

This retrospective study was conducted on 48 UBC specimens, which retrieved from the archives of Pathology Departments in El Demerdash Hospital and Ain Shams Specialized Hospital, Ain Shams University spanning the period between January 2017 and June 2021. The studied cases included 48 cases of UBC received as 11 cystectomy specimens and 37 cystoscopic biopsy. All clinicopathological data are tabulated in Table 1.

Table 1: Clinicopathological parameters

AGE				
Mean (SD)	64.7 (9.7)			
Median	64			
Range	30-86			
Variables	Number		Percent (%)	
Gender				
Male: female ratio=11:1				
Female	4		8.33	
Male	44		91.67	
Type of specimens				
Radical Cystectomy	11		22.9	
Cystoscopy	37		77.1	
Histologic type				
S.C.C	4		8.3	
Urothelial carcinoma:	44		91.7	
Noninvasive carcinoma				
N/TCC	3/44		6.8	
N/total	3/48		6.3	
Invasive carcinoma				
N/TCC	41/44		93.2	
N/total	41/48		85.4	
Grading				
SCC	N/SCC	N/total	N/SCC	N/total
G1	0/4	0/48	0	0
G2	3/4	3/48	75	6.25
G3	1/4	1/48	25	2.1
TCC	N/TCC	N/total	N/TCC	N/total
Low grade	17/44	17/48	38.6	35.4
High grade	27/44	27/48	61.4	56.25
TNM stage				
T				
T1	9/20		45	
T2	3/20		15	
T3	6/20		30	
T4	2/20		10	
Early stage (T1+T2)	12/20		60	
Late stage (T3+T4)	8/20		40	
Lymph node status				
Positive	3		6.3	
Negative	6		12.5	
could not be assessed	39		81.3	
Muscle propria invasion				
Positive	28		58.3	
Negative	9		18.7	
Could not be assessed	11		22.9	
Margin (Urethral and ureteric)				
Positive	3		6.3	
negative	8		16.7	
Could not be assessed	37		77.1	
Lymph vascular invasion				
Positive	5		10.4	
Negative	41		85.4	
suspicious	2		4.2	
Necrosis				
present	6		12.5	
absent	42		87.5	
Perineural invasion				
Positive	4		8.3	
Negative	44		91.7	
Bilharziasis				
Present	10		20.8	
Absent	38		79.2	

Inclusion criteria

Selection of cases was meant to include different tumor grades of UBC and in balanced comparable groups to serve the aim of this study.

Exclusion criteria

There was an exclusion of UBC cases lacking archival data, minimally representative tissue biopsies, as well as patients who had received prebiopsy chemotherapy.

The study methodology

- Clinical data collection: Clinical data regarding age and gender were collected from the patient's medical archives
- Histopathological assessment: 4 μm thick were cut from each block, stained with routine H. and E. to confirm the diagnosis, and assure histopathologic evaluation of tumor characteristics (Grade, stage, Bilharzial background, lymphovascular invasion, perineurial invasion, lymph node status, margin status, and presence of necrosis) according to the latest WHO classification [2], AJCC guidelines [7].

Immunohistochemical study

Immunohistochemistry (IHC) was done using streptavidin-biotin amplified system. The primary antibody TGFB1 is IgG purified Rabbit Polyclonal antibody (bs-0086R, Biossusa, USA). It was received as 100 μl with 1 μg/μl concentration and diluted by phosphate buffer saline in a dilution 1:100. was performed. An automated Universal Dako cytomation Labeled streptavidin-Biotin-2 system and Horseradish Peroxidase (LSAB-2 System, HRP Kit, Catalogue No. k0679) were used. Normal bone was used as a positive control and the staining process was done according to manufacturer manual.

Immunostaining interpretation

Expression was evaluated in all studied sections in CAFs and interpreted as follows (modified from Kim *et al*., 2001) [8].

- Low expression: When TGFB1 expression is expressed in less than 10% with any intensity in any compartment
- High expression: When TGFB1 is expressed in more than 10% with any intensity.

Statistical analysis

Data were collected, tabulated, and statistically analyzed using a personal computer with the Statistical Package for the Social Sciences version 22 program. Tables were analyzed with the following tests.

Descriptive statistics

Arithmetic mean (x), standard deviation (SD), percentage (%), range, and median.

Analytical statistics

Fisher's exact test and Chi-square, McNemar test.

Results

The present study included 48 bladder carcinoma cases managed by cystectomy (11 cases) in addition to cystoscopy biopsies (37 cases), in both varieties, specimens were not preceded by chemotherapy. The clinicopathological data are summarized in Table 1.

Immunohistochemical results of TGFB1 expression in CAFs

High TGFB1 expression was identified in 28/48 (58.3%) while low expression was identified in 20/48 (41.7%). Expression was localized mainly in the cytoplasm (Figures 1 and 2).

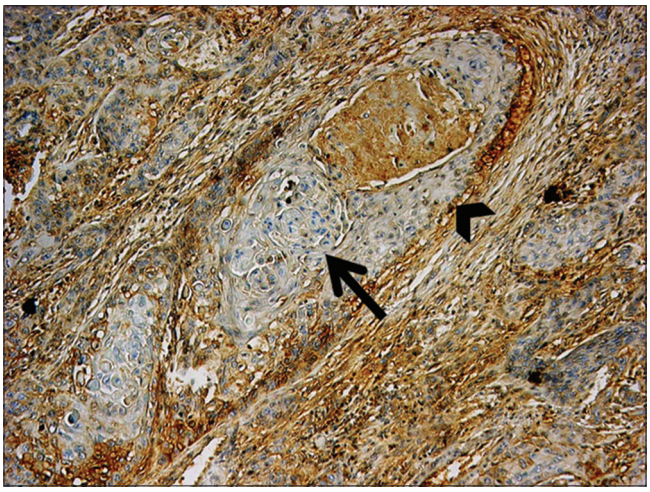

Figure 1: A case of squamous cell carcinoma showing tumor cells are negative to TGFB1 (black arrow), but they are shielding themselves by CAFs which strongly express TGFB1 (arrow head) (IHC ×100)

Regarding tumor type TGFB1 was highly expressed in squamous cell carcinoma and Urothelial carcinoma with special differentiation (e.g., with squamoid and or glandular differentiation), while in classical Urothelial carcinoma, the expression was low ($p \leq 0.02$). Moreover, high expression was significantly associated with high tumor grade ($p = <0.001$), presence of muscle invasion ($p \leq 0.001$), and higher tumor stage ($p \leq 0.01$). The presence of preceding bilharziasis also correlated with high TGFB1 expression ($p \leq 0.003$), and presence of necrosis ($p \leq 0.03$ S). The rest of pathological data showed no significant expression in the CAFs.

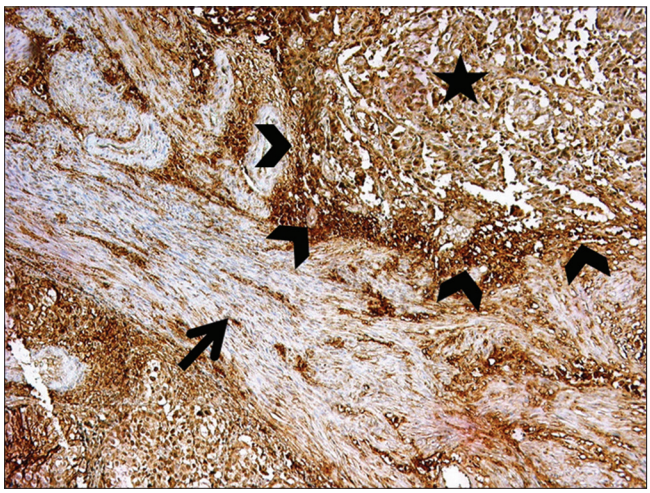

Figure 2: A case of invasive urothelial carcinoma with squamoid differentiation showing strong expression of TGFB1 in both the tumor cells (Asterix) as well as the CAFs mainly those bordering tumor cells (arrow heads) rather than those nearby (black arrow) (IHC ×100)

Immunohistochemical results of TGFB1 expression in both CAFs and in the tumor cells

TGFB1 expression in both tumor cells and CAFs was unrelated to each other among the studied tumor groups ($p = 0.002$) (Table 2).

Additional subtle yet important observations regarding aberrant TGFB1 expression

In muscularis propria

All non-invaded muscularis propria were negative to TGFB1, 48/48 (100%) except for epimysium. Nonetheless, the frontier UBC cells that were progressing to muscularis propria invasion were always bestowed by a TGFB1 CAFs niche along their growing edge (Figure 3).

In carcinoma in situ

High expression of TGFB1 was observed in all the three carcinoma *in situ* (CIS) among tumor cells as compared to absent TGFB1 expression in all sampled non-neoplastic urothelium (Figure 4).

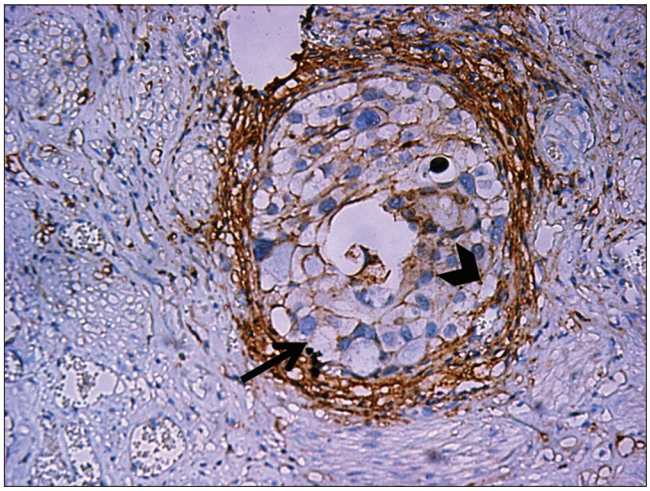

Figure 3: A ball of the tumor cells invading the muscularis propria showing moderate membranous expression of TGFB1 (arrow head) in contrast to strong expression of the CAFs shield (black arrow) in a case of high-grade invasive urothelial carcinoma (IHC ×400)

Spatial distribution

We noticed an evident spatial variation in staining intensity; being the strongest along the tumor progressing edge, where it invades the muscle and fat in contrast to an obviously weaker intensity elsewhere in the rest of tumor section (Figure 5).

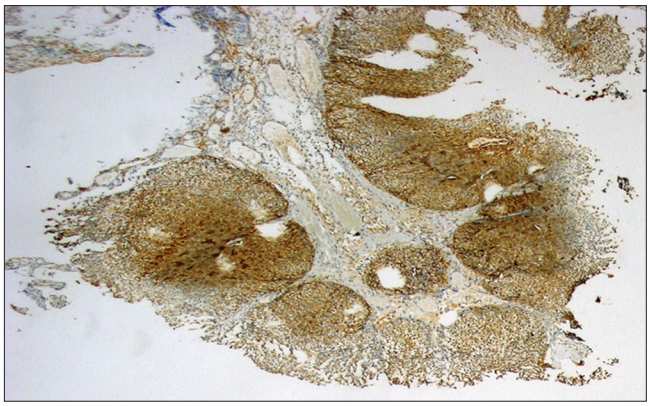

Figure 4: Carcinoma in situ with strong expression of TGFB1 in tumor cells in a case of papillary urothelial carcinoma – Negative/ low expression of TGB1 in uninvaded underlying connective tissue (IHC ×40)

Discussion

Urothelial carcinoma of the UB obviously still poses an important toll in cancer burden, being a prominent contributor in both cancer morbidity and mortality, it represents a rich arena for research [1]. The ingrowing knowledge about the role of TME had motivated a plethora of research studies to focus on launching new microenvironment targeted therapeutic candidates. Although new treatments against TGFB1 are emerging, yet the expression in UBC CAFs had not been studied [6]. Hence, we dedicated this study for TGFB1 expression in CAFs in UBC.

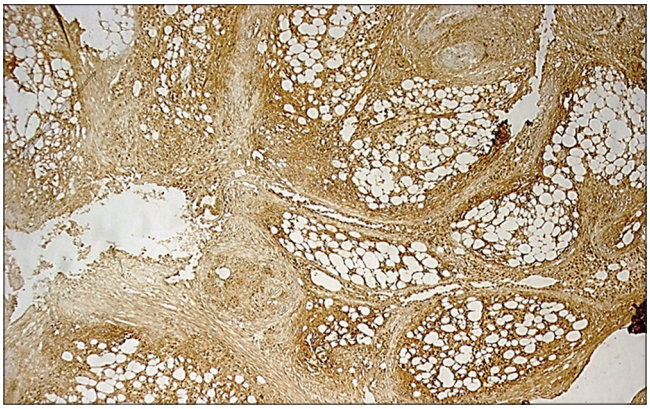

Figure 5: A case of invasive poorly differentiated urothelial carcinoma infiltrating the fat. The tumor cells show spatial distribution of TGFB1 that the expression was stronger in the growing boundary toward the infiltrated fat than other parts of the tumor. The CAFs show high expression as well (IHC ×100)

In contrast to the pre-established weak staining pattern of normal UB stromal cells [9], this novel study demonstrates a significantly altered TGFB1 expression in TME related CAFs in UBCs.

There was significant relation between TGFB1 expression in CAFs and tumor type, high tumor grade, presence of muscle invasion, higher stage, presence of preceding bilharziasis, and necrosis. This statistical significance reflects that CAFs employ TGFB1 for growth of the tumor, invasion, and metastasis. As far as we know, this study is the first of its kind to study the expression of TGFB1 in CAFs in UBC by IHC and correlate it with clinicopathological data. However, TGFB1 was studied in other organs such as papillary thyroid carcinoma (PTC), where expression of TGFB1 was reported to be significantly higher in the stroma of PTC than in nodular goiter and normal thyroid tissues; moreover, higher expression of TGFB1 was closely related with lymph node metastasis, but not with advanced tumor stages [10].

We spotted that when tumor cells are penetrating the muscularis propria, they bestow themselves by niche of CAFs that strongly express TGFB1 forming a layered ball. Putting into consideration the proven role of CAFs niche in supporting growth, migration, and invasion of tumor cells, it is rational to conclude that such bordering of CAFs with high expression of TGFB1 likely generates a hospitable environment for tumor progression. Creating a niche that promote tumor progression, metastasis, and therapeutic resistance is certainly achieved through reprogramming and remodeling the TME [11], [12]. Our observation emphasizes that TGFB1 expressing CAFs are most likely involved in such priming setting. A reminiscent context is also recapitulated in the pre-metastasis niches (PMNs), where a tumor-promoting microenvironment must be set to embrace metastasizing cancer cells recruits in their new residence. It is plausible to assume that TGFB1 expression by CAFs extend their

support to cancer cells in their metastasizing destination given the fact that CAFs enter the blood circulation and migrate to the targeted organs to form the new niche. Moreover, TGFB1 secreted by CAFs and tumor cells in the primary lesion is one of the soluble factors promoting PMN [13], [14].

As far as we know, this is the first study to correlate between TGFB1 expression in CAFs and tumor necrosis. Our results proved a significant correlation among TGFB1 expression and presence of necrosis. Assessment of tumor necrosis in urinary tract proved to be an indicator of aggressive biology and adverse clinical outcomes and could affect the decision of treatment. Thus, some authors suggested that evaluation of extensive tumor necrosis should be a part of standard pathologic reporting [15], [16]. Tumor necrosis is associated with hypoxia, angiogenesis and inflammation responses through HIF1A, NF-kB, and PI3K/mTOR pathways [17]. Not surprisingly, mTOR is activated by TGFB1, it drives epithelial–mesenchymal transition and cell invasion [18]. Whether these scenarios are behind the high expression of TGFB1 in partially necrotic UBCs in our study or not can't be fully verified in the scope of our study; nevertheless, it introduces valuable insights about UBCs biology.

Furthermore, we found that TGFB1 expression in CAFs was significantly increased when cancer is associated with Schistosomiasis (p = 0.003). Schistosomiasis is one of the major predisposing factors for UBCs within the Nile basin region, the mechanisms by which this inflammatory arena is hi-jacked to a carcinogenic track are yet elusive [19]. Our study may contribute to unleashing these ambiguities by implying that TGFB1 mediates Schistosomal cancer predisposition by stimulating CAFs to endow a tumor potentiating microenvironment alluring epithelial cells to acquire an aggressive behavior.

Unexpectedly, TGFB1 expression in both tumor cells and CAFs was unrelated to each other among the studied tumor groups (p = 0.002).

Additional to the statistically analyzed results of our study, we raise some of our critical observations assuming their potentially promising insights that might inspire verification through other research studies of larger scope and specified focus; first and foremost, we noticed an evident spatial variation in staining intensity; being the strongest along the tumor progressing edge, where it invades the muscle and fat in contrast to an obviously weaker intensity elsewhere in the rest of tumor section. Thus, we suggest that there is spatial distribution of the TGFB1 throughout the tumor reflecting the pivotal role in the vanguard of tumor invasion.

Indeed, this variation might have contributed to some of the contradictory results in some of our MIBC cases, because assessment protocol dictated the overall impression of staining percentage of tested tumor section regardless of its spatial localization. Moreover, such remark further poses the inquiry of whether TGFB1 expression is a temporary step specified status, that is, later silenced or a rather stable acquired phenotype that lasts once gained.

Last but not least, TGFB1 expression in CIS was diffuse, homogenous, and strong in all urothelial layers in contrast to normal urothelium, in which a strikingly stronger immunostaining was seen in both superficial and basal cell layers than the intermediate cell layers [9]. Such observation might provide a valuable diagnostic utility in differentiating CIS from non-neoplastic urothelium, but more importantly may reflect possible predictive value of TGFB1 assessment in non-invasive urothelial lesions.

Table 2: Correlation between the immunohistochemistry marker in tumor and fibroblasts

Expression in tumor	Expression in CAFS						p-value*
	Low		High		Total		
	n	%	N	%	n	%	
Low	0	0.0%	4	100.0%	4	100.0%	0.002 HS
High	20	45.5%	24	54.5%	44	100.0%	

*McNemar test

Ethical consideration

This study abided to the revised tenets of the Declaration of Helsinki.

The Institutional Ethical Committee approval of Ain Shams University hospitals (FWA 000017585) was obtained in alignment to patients' rights in full anonymity of their clinical and pathological data through a strict numerical coded archival recruitment system.

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