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ORIGINAL PAPER

Increased expression of transglutaminase-2 is associated with invasive disease in bladder cancer

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Summary

Purpose: *Transglutaminase-2 is associated with tumor invasion, metastasis development, chemoresistance and poor prognosis in various cancer types. In this study, our aim was to show the association between increased transglutaminase-2 expression and the invasive pattern of bladder cancer.*

Materials and methods: *Tumor tissues from eighty-eight patients with bladder cancer (43 muscle-invasive, 45 non-muscle invasive bladder cancer) were immunohistochemically evaluated for TG2 expression.*

Results: *Transglutaminase-2 expression score was higher in muscle- invasive bladder cancer compared to non-muscle invasive bladder cancer tissues (5.37 ± 1.5 vs. 0.71 ± 1.4, p < 0.001). No statistically significant difference was found in transglutaminase-2 expression scores between metastatic and non-metastatic disease in MIBC group. Different tumor and lymph- node stages in MIBC were also found to be not correlated with transglutaminase-2 expression scores.*

Conclusions: *The over-expression of transglutaminase-2 is associated with invasive disease in bladder cancer. According to our results, transglutaminase-2 has the potential to be useful for predicting the invasion in bladder cancer and addressing treatment.*

KEY WORDS: *Transglutaminase-2; Muscle-invasive bladder cancer; Malignant neoplasm; Tumor biomarker.*

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INTRODUCTION

Bladder cancer (BC) is the 10th most common cancer worldwide, and its incidence is rising, especially in developed countries. Bladder cancer is more common in men than women, with lifetime risk of 1 to 27 in men and 1 to 89 for women, respectively (1). The advanced age is now recognized as the greatest risk factor for bladder cancer, with the average age of BC diagnosis being 70 years (2). Tobacco consumption is the most important modifiable risk factor for bladder cancer, accounting nearly 50% of all cases. Urothelial carcinoma is the most common subtype of BC (3).

Bladder cancer is divided into two main categories: *non-muscle invasive bladder cancer* (NMIBC), which is confined to the mucosa (Ta and carcinoma in situ "CIS") or lamina propria (T1), and tumors that invade the muscle (T2) and beyond (T3, T4) which are defined as *muscle- invasive bladder cancer* (MIBC). Approximately 75% of newly diagnosed BCs are found at the non-muscle invasive stage. NMIBCs are characterized by favorable survival rates and with progression rates varying between 6-17%, depending on the T stage of the tumor (Ta-CIS- T1) (4). However, up to 67% of all patients with NMIBC may experience disease recurrence. In contrast, in MIBC, five-year survival rates drop to 60% for localized disease and below 10% for metastatic disease (5). Efforts are being made to identify new markers for BC to predict invasive potential and prognosis of the disease.

Transglutaminases are an enzyme family that perform multiple calcium dependent posttranslational modifications of proteins. Tissue transglutaminase also known as transglutaminase- 2 (TG2), is the most abundant member of this family and it can be found in all cellular compartments (nucleus, cytoplasm, organelles) as well as in the extracellular space (6). In cellular environment, TG2 is involved in various enzymatic activities such as transamidation, GTP/ATP hydrolyse, kinase, proteolytic and scaffolding. Through these activities, TG2 plays essential roles in diverse physiological processes, including cell growth and differentiation, adhesion, migration, angiogenesis, autophagy, extracellular formation, apoptosis (7). The oncogenic potential of TG2 has been demonstrated in various cancer types such as breast carcinoma, pancreatic adenocarcinoma, colorectal carcinoma and central nervous system tumors in previous studies (8-11). Resistance to systemic drugs and development of metastases together account for nearly 90% of all cancer-related deaths. Identifying of biomarkers that can promote tumor invasiveness, drug resistance and metastasis is an important goal, as these proteins can be helpful in estimating the prognosis of the disease and may serve as potential targets for the therapy. In carcinogenesis, TG2 has shown to play various roles. Firstly, TG2 can promote *epithelial- to mesenchymal transition* (EMT), which is believed to be the first step of tumor progression and metastasis (12). TG2 also contributes to chemoresistance and promote the acquisition of cancer stem-cell like properties of the tumor cells by activating NF-κB and PI3K/Akt pathway (13). Additionally, TG2 influ-

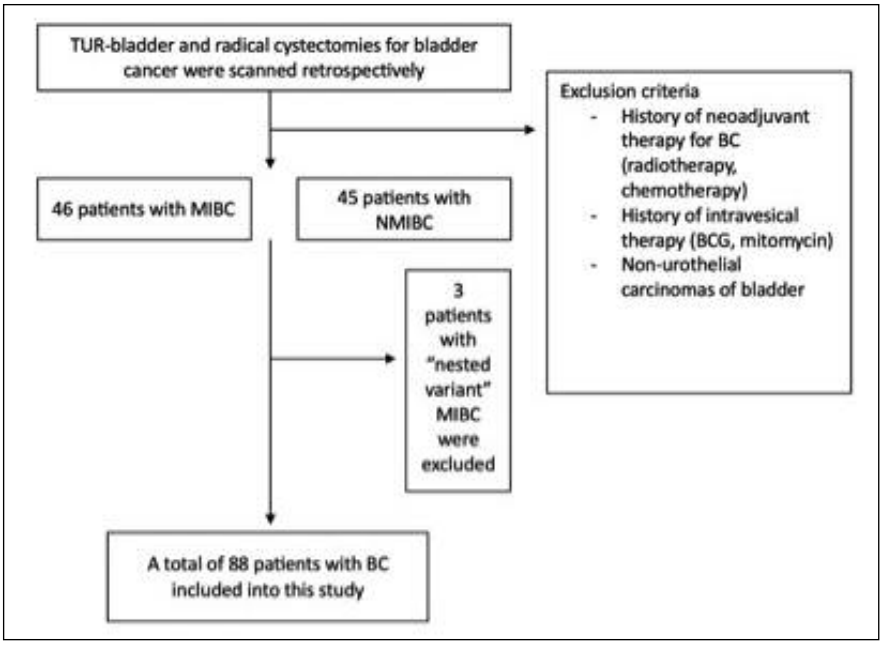
ences the tumor micro-environment by cross-linking extracellular matrix proteins, thereby facilitating tumor- ECM interaction. Through these mechanisms, TG2 has shown to be associated with increased cancer cell adhesion, migration, metastasis and invasion in different cancer types (14). In this study, our aim was to evaluate the role of transglutaminase-2 in bladder cancer.

Materials and methods

After the approval of local ethics committee (No: 2021/514/202/5), cases dated between January 2014 and May 2021 involving transurethral resections and radical cystectomies for bladder cancer in our Urology department database were retrospectively scanned. All demographic characteristics, clinical and pathological parameters were evaluated. A total of 46 patients with MIBC were identified for the study group and 45 patients with NMIBC were selected as the control group. The control group were consisted of patients with demographic characteristics similar to the study group. Exclusion criteria were: history of neoadjuvant therapy for BC, history of intravesical therapy and non-urothelial carcinomas of bladder (Figure 1). A total of 91 patients were included into this study.

Paraffin-embedded cancer tissues of these patients were extracted from Pathology Department's archives.

Figure 1.
PRISMA diagram showing the selection of the patients.



Immunohistochemical evaluation

Formalin fixed paraffin-embedded tissues were used for immunohistochemical evaluation. After the inspection of all hematoxylin-eosine-stained slides of the tumor samples, a single paraffin-embedded block with the most evident tumor tissue was selected for each case. Tissue sections obtained from the paraffin blocks were fumigated for one day. The next day, the slides were placed in a Benchmark XT/IHC system staining module (*Roche Diagnostics, Basel, Switzerland*). Deparaffinization, antigen retrieval with EDTA, antibody incubation (*Transglutaminase-2 CUB 7402, Invitrogen, Massachusetts, USA, MA-12739, 1/200 dilution, 32 minutes incubation*), amplification and counter staining with HE processes were performed respectively. After these steps, slides were washed, dried and sealed.

Microscopic assessment

According to the manufacturer's manuals, tumor samples from an invasive ductal breast adenocarcinoma case were selected as control tissue. Efficacy and the reaction conditions of the antibody were determined by using this control tissue.

The assessments of the tumor tissues were performed by SK, who is an experienced uro-pathologist with the participation of one of the urologists (YK) as an observer. Both evaluators were blinded to the clinical and pathological features of the patients.

All tumor slides were evaluated with microscope under 4x, 10x, 20x, 40x and 100x magnifications when necessary. Based on previous studies (15), cytoplasmic and membranous staining of the cells was assessed and according to findings in 4x, a semi-quantitative chart was designed. Staining percentage was scored between 0-4 points (< 10%-0 points, 10-30% 1 point, 30-50%-2 points, 50-70%-3 points and > 70%-4 points) and staining intensity was scored between 0-3 points (no staining- 0 points, weak-1 point, moderate-2 points and strong- 3 points) with maximum total score of 7 points (Table 1).

Three patients with "*nested variant of urothelial carcinoma*" from MIBC group were excluded from the study due to a lack of staining with

Table 1.
The semi-quantitative chart for transglutaminase-2 antibody staining scoring system.

Staining parameter	Points
Staining percentage (% score)	
< 10%	0
10-30%	1
30-50%	2
50-70%	3
> 70%	4
Staining intensity (intensity score)	
No staining	0
Weak	1
Moderate	2
Strong	3

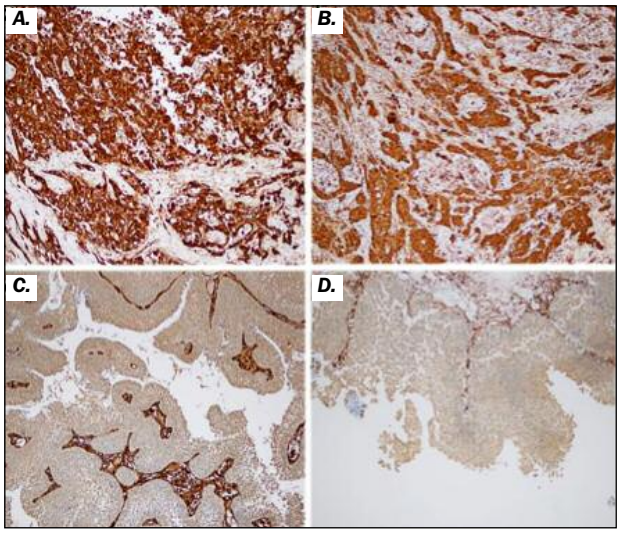


Figure 2.
Microscopic images of immunohistochemical TG2 staining of the tissues.
A. *57 years old man, MIBC, pT3b, % score: 4/intensity score: 3 total score: 7.*
B. *68 years old woman, MIBC with sarcomatoid differentiation, pT4, % score: 4/intensity score: 3 total score: 7.*
C. *44 years old man, low grade NMIBC, pTa, % score: 0/intensity score: 0 total score: 0.*
D. *51 years old man, low grade NMIBC, pTa, % score: 0/intensity score: 0 total score: 0.*

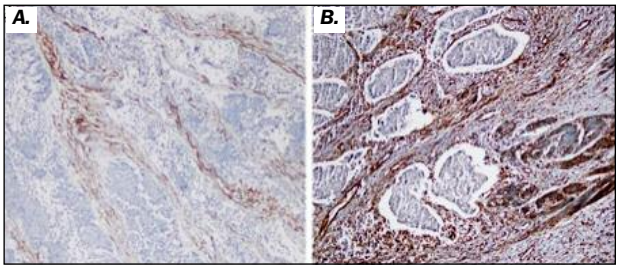


Figure 3.
Microscopic images of immunohistochemical TG2 staining of the tissues.
A. *63 years old man, MIBC with nested variant, T2, % score: 0/intensity score: 0 total score: 0.*
B. *54 years old man, MIBC, T3a, % score: 2/intensity score: 1 total score 3.*

the antibody. A total of 88 patients with BC were included into this study.
The microscopic images of immunohistochemical TG2 antibody staining of the tissues are presented in Figure 2 and Figure 3.

Statistical analysis

Statistical analysis was performed with IBM SPSS software (*ver. 26 for MacOS, IBM, USA*). Distribution of the variables was measured by Kolmogorov-Smirnov test. All demographic, clinical and histopathological parameters were analyzed using Kruskal-Wallis test, Mann-Whitney U test and Chi-square test. A p value < 0.05 was considered as statistically significant.

Results

A total of 88 patients were included in this study. Group 1 consisted of 43 MIBC patients (40 men, 3 women), and Group 2 consisted of 45 NMIBC patients (41 men, 4 women). Three patients with nested variant of MIBC were excluded (Figure 1). The average age was 63.4 ± 10.3 years in Group 1 and 61.1 ± 11.9 years in Group 2 (p = 0.447). All 88 patients were diagnosed with urothelial carcinoma of the bladder. All patients in Group 1 had high-grade carcinoma, while 3 patients (6.6%) in Group 2 had high-grade carcinoma (Table 2).
The average transglutaminase-2 (TG2) staining score was significantly higher in Group 1 compared to Group 2 (5.37 ± 1.5 *vs.* 0.71 ± 1.4, p < 0.001) (Table 3).
Further analysis of patients with MIBC (Group 1) was

Table 2.
Demographic, clinical and pathological parameters of two groups.

Group no	Group 1 (MIBC group)	Group 2 (NMIBC group)
Patient number	43	45
Age	63.40 ± 10.3	61.13 + 11.9
Sex (n, %)		
Men	40 (93%)	41 (91.1%)
Women	3 (7%)	4 (8.9%)
Tumor histology (n, %)	Urothelial carcinoma - 43 (100%)	Urothelial carcinoma - 45 (100%)
Tumor stage (n, %)		
pT2	16 (37%)	
pT3	15 (34.8%)	Ta - 45 (100%)
pT4	12 (27.9%)	
Lymph node involvement (n, %)		
N0	18 (41.8%)	N0 - 45 (100%)
N1	12 (27.9%)	
N2	11 (25.5%)	
N3	2 (4.6%)	
Tumor grade (n, %)	High grade - 43 (100%)	Low grade - 42 (93.3%) High grade - 3 (6.6%)

Table 3.
The comparison of mean transglutaminase-2 staining scores of two groups.

	Group 1 (MIBC group)	Group 2 (NMIBC group)	P value
TG2 staining score (mean ± SD)	5.37 ± 1.5	0.71 ± 1.4	< 0.001

Table 4.
The comparison of mean transglutaminse-2 scores in metastatic and non-metastatic MIBC patients.

	Present (Mean TG2 score + SD, number)	Not present (Mean TG2 score + SD, number)	P value
Metastasis	5.39 ± 1.6 (n: 28)	5.33 ± 1.4 (n: 15)	0.824 *
* *Mann Whitney- U test.*			

Table 5.
The comparison of mean transglutaminse-2 scores in MIBC patients subgroup regarding to T and N stages.

	Mean TG2 score (number)	P value
T stage		
2	5.31 ± 1.44 (n: 16)	0.142 **
3	5.93 ± 1.38 (n: 15)	
4	4.75 ± 1.71 (n: 12)	
N stage		
0	5.33 ± 1.41 (n: 18)	0.905 **
1	5.25 ± 1.71 (n: 12)	
2	5.73 ± 1.34 (n: 11)	
3	4.5 ± 3.53 (n: 2)	
** *Kruskal- Wallis test.*		

conducted to identify factors potentially influencing TG2 expression. No statistically significant difference in TG2 staining scores was observed between metastatic and non-metastatic MIBC patients (5.39 ± 1.6 *vs.* 5.33 ± 1.4, p = 0.824) (Table 4). Additionally, there was also no statistically significant difference in TG2 staining scores between tumors at different tumor stages (p = 0.142) or lymph node stages (p = 0.905) (Table 5).
Interestingly, three patients with the nested variant of MIBC showed no staining with the transglutaminase-2 antibody.

Discussion

Our study reveals a significant upregulation of *transglutaminase-2* (TG2) in MIBC compared to NMIBC (5.37 ± 1.5 *vs* 0.71 ± 1.4, p < 0.001). While TG2 expression was significantly elevated in invasive tumors compared to non-invasive tumors, it showed no correlation with lymph node involvement, tumor stage, or metastatic status in within the MIBC subgroup, highlighting its presence across all stages of MIBC without significant variations. Interestingly, the absence of TG2 staining in the nested variant MIBC cases highlights potential histological variability, suggesting that TG2 expression is not uniform across all bladder cancer variants.
One of the key ways TG2's contributions to cancer progression is by promoting *epithelial-to-mesenchymal transition* (EMT) of cancer cells. EMT is a major pathway of the tumor cells during cancer progression (16). TG2 activates cancer cells EMT through FAK, Akt and NF-kB pathway (17). During EMT, epithelial cells lose their cell-to-cell adhesion and take on a more mobile, mesenchymal-like state. This leads to the loss of epithelial markers like E-cadherin and an increase in mesenchymal markers such as vimentin and fibronectin. These changes enable cancer cells to become more migratory and invasive, significantly increasing their potential of invasion and forming distant metastases. Secondly, TG2 overexpression has been linked to the acquisition of stem-cell like properties of the cancer cells which is associated with increased tumor-initiating capacity and chemoresistance (18). Additionally, in carcinogenesis TG2 modulates the tumor microenvironment by crosslinking ECM proteins and enhances cell adhesion, migration and tumor survival.
When compared to other studies, our findings are coherent with prior observations that link TG2 expression to tumor aggressiveness in various malignancies. In bladder cancer, *Hager et al.* observed that TG2 is primarily expressed in grade 3 and 4 muscle- invasive tumors, while it remains undetectable in normal urothelium and low-grade papillary tumors. Their study further demonstrated a lack of TG2 staining in distant solid organ metastases, suggesting TG2's role may be limited to the local invasive phase rather than systemic metastatic disease. In breast cancer, *Kumar et al.* showed increased expression of TG2 induces EMT and stem-cell properties in mammary epithelial cells therefore promoting aggressive disease and metastasis (19). In a study by *Shinde et al.* it was shown that TG2 contribute to resistance to chemotherapy in HER2 positive breast cancer cells through the activation of the NF-κB signaling pathway (20). In a review, *Li et al.* showed that increased TG2 expression is elevated in lung and bone metastasis and TG2 can mediate multiple therapy resistance to chemotherapeutic drugs and immune check-point inhibitors by modulating tumor microenvironment and promoting EMT and CSC-like properties (21).
In colorectal cancer, increased expression of TG2 has shown to be associated with lower *disease-free survival* (DFS), *overall-survival* (OS) and earlier relapse (22). In an invitro study, overexpression of TG2 in colorectal cancer stem-cells has shown to be associated with increased metastatic potential via EMT pathway and TG2 knockdown reversed the EMT proving the potential of TG2 as a therapeutic target for aggressive CRC (23).
In ovarian cancer cells, TG2 binds directly to fibronectin and stabilizes extracellular matrix by enhancing fibronectin- integrin B-1 complex. By activating *integrin-linked kinase* (ILK), TG2 favors cancer-cell adhesion, extracellular matrix reorganization, migration and metastatic spread. Statistical analyses showed high expression of TG2 and ILK is associated with lower overall-survival in ovarian cancer. In-vitro knockdown of TG2 demonstrated significant reduction of cancer cell adhesion and metastatic potential and hence showing the potential role of TG2 as a therapeutic target for ovarian cancer (24).
In *non-small-cell lung cancer* (NSCLC), higher TG2 expressing tumors have found to be associated with more advanced stage/metastatic disease, increased chemotherapy resistance, lower DFS, poorer OS compared to lower TG2 expressed tumors (25). It has also reported that inhibition of TG2 in lung cancer increase radiosensitivity of tumor cells (26). Corelated with these results, TG2 may serve as a prognostic factor in lung cancer. In pancreatic cancer, tumor with higher TG2 expressions were found to have

poorer clinicopathologic features (higher nodal metastasis, advanced clinical stage, less chemotherapy sensitivity, increased tumor invasion and metastatic potential) (11).
In this study we were able to detect higher expression of TG2 in muscle invasive bladder cancer compared to non-muscle invasive bladder cancer. Our results were corelated with previous findings about the potential roles of transglutaminase-2's in advanced malignancy in various cancer types. But our study is not without some limitations. Firstly, the small number of samples is the main limitation of this study. Secondly, clinical data of the patients are not obtained thus no commentary can be made on the effects of transglutaminase-2 on overall-survival and disease-free survival rates in bladder cancer. Nevertheless, considering that muscle-invasive BC has lower overall- survival and disease-free survival compared to NMIBC, higher TG2 can be held responsible for worse prognosis in bladder cancer. However, further clinical data are necessary for this purpose. On the other hand, our study is one of the very few studies on this subject and our results showed clearly that TG2 expression is stronger in invasive bladder cancer tissues compared to non-invasive tumors. Our findings may form a basis for further clinical studies.

Conclusions

In this study, our data showed that higher expression of transglutaminase-2 is associated with increased invasion potential in bladder cancer. According to our results, transglutaminase-2 has the potential for predicting prognosis of bladder cancer and being a therapeutic target.

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Declarations

Ethical approval and consent for participate: *This study was approved by the local ethical committee (No: 2021/514/202/5). All patients consented for participation.*

Consent for publication: *All patients consented for publication.*

Availability of data and material: *All data and material of this study is available for further assessment from the corresponding author.*

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