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MUSCLE-INVASIVE TRANSITIONAL CELL CARCINOMA OF THE URINARY BLADDER IS ASSOCIATED WITH DOWN-REGULATED CPP32 EXPRESSION AND BCL-2 POSITIVITY

INVAZIVNI KARCINOM PRELAZNOG EPITELA MOKRAĆNE BEŠIKE POKAZUJE SMANJENU EKSPRESIJU CPP32 I POVEĆANO PRISUSTVO BCL-2

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Summary: The objective was to get insight into the role of executive apoptotic enzyme caspase 3 (CPP32) and regulatory antiapoptotic protein Bcl-2 in the malignant phenotype of TCC. Samples were obtained from 84 TCC patients, who underwent transurethral resection, partial or radical cystectomy. Staging showed a superficial growth pattern in 41 patient, while other 43 showed invasive characteristics. Expression of CPP32 and Bcl-2 was determined by immunocytochemistry. Levels of expression were correlated with tumor stage and grade. Expression of CPP32 was positive in 80% of TCC patients. Low-, medium- and high positive status were observed in 18%, 24% and 38% of patients, respectively. There was a signifficant difference in the CPP32 expression between groups with superficial and invasive TCC tumors ($p = 0.032$), with frequency of CPP32 negative samples being higher and CPP32 high-positive samples being lower in patients with muscle-invasive tumors. Significant association was also found between CPP32 expression and tumor stage ($p = 0.043$). The positive rate of Bcl-2 protein expression was 48%. There was a statisticaly signifficant difference in the rate of Bcl-2 positivity between superficial and invasive TCC ($p = 0.005$), with frequency of Bcl-2 positive patients being higher in muscle-invasive TCC. Significant association was also found between Bcl-2 expression and both tumor grade ($p=0.032$) and stage ($p=0.007$). Muscle invasive TCC of the urinary bladder is associated with

Kratak sadržaj: Ispitivana je uloga izvršnog molekula apoptoze, kaspaze 3 (CPP32) i regulatornog antiapoptotskog proteina Bcl-2 u malignom fenotipu karcinoma prelaznog epitela mokraćne bešike. U istraživanju su korišćeni uzorci tumorskog tkiva mokraćne bešike 84 bolesnika sa karcinomom prelaznog epitela, koji su podvrgnuti transuretralnoj resekciji, parcijalnoj ili radikalnoj cistektomiji. Na osnovu stepena invazivnosti, uzorci su podeljeni u dve grupe: 41 neinvazivni tumor i 43 invazivna tumora. Ekspresija CPP32 i Bcl-2 određivana je metodom imunocitohemije. Ispitivana je povezanost nivoa ekspresije sa stepenom maligniteta i stadijumom tumorske bolesti. Ekspresija CPP32 bila je pozitivna u 80% uzoraka tumorskog tkiva pacijenata sa karcinomom prelaznog epitela. Slabo pozitivno bojenje je uočeno u 18%, umereno pozitivno u 24%, a veoma pozitivno u 38% pacijenata. Uočena je statistički značajna razlika u ekspresiji CPP32 između neinvazivnih i invazivnih karcinoma ($p=0,032$), pri čemu je učestalost CPP32 negativnih pacijenata bila veća, a CPP32 veoma pozitivnih pacijenata manja u invazivnim karcinomima. Značajna povezanost je uočena i između smanjene ekspresije CPP32 i stadijumima tumora ($p=0,043$). Prisustvo antiapoptotskog Bcl-2 proteina je utvrđeno u 48% uzorakak tumorskog tkiva. Statistički značajna razlika u učestalosti Bcl-2 pozitivnih pacijenata je uočena između neinvazivnih i invazivnih tumora ($p=0,005$), pri čemu je učestalost Bcl-2 pozitivnih pacijenata bila veća u grupi invazivnih

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down-regulated expression of CPP32 and Bcl-2 positivity. Down-regulation of CPP32 and up-regulated Bcl-2 might, at least partially, play a role in the development of invasive characteristics of TCC.

Keywords: CPP32, Bcl-2, apoptosis, TCC, urinary bladder

karcinoma. Statistički značajna razlika je uočena između ekspresije Bcl-2 i stepena maligniteta (p=0,032), kao i stadijuma tumorske bolesti (p=0,007).

Ključne reči: CPP32, Bcl-2, apoptoza, invazivni karcinom prelaznog epitela, mokraćna bešika

Introduction

Bladder cancer is the second most frequent genitourinary tumor and a significant cause of morbidity and mortality (1). Transitional cell carcinoma of the urinary bladder (TCC) represents more than 90% of all bladder carcinomas (2). The treatment of choice for muscle-invasive TCC is radical cystectomy and bilateral pelvic lymph node dissection, still approximately 50% of all patients with muscle-invasive TCC develope metastatic disease within 2 years of cystectomy (1, 3). Therefore, elucidation of the molecular changes leading to the development of muscle-invasive TCC and analysis of new parameters that could predict more agressive behaviour of these tumors are a necessity. Since mounting evidence exists that alterations in the cascades of apoptotic and pro-survival signals might be involved in the development of various human tumors (4, 5), the expression of regulatory and executive apoptotic molecules in TCC has also gained some attention. However, the available data describing the role of apoptotic molecules in TCC of the urinary bladder are both limited and conflicting.

Among the members of apoptotic cascade in TCC, expression of the Bcl-2 oncoprotein has been mostly studied. The Bcl-2 oncoprotein is an anti-apoptotic member of the Bcl-2 family, that is mainly located in mitochondrial membranes and to a lesser extent in the endoplasmatic reticulum and nuclear envelope (6). By preventing cytochrome c release from mitochondria and further proteolytic activation of proteolytic enzymes caspases, it inhibits apoptosis. That way Bcl-2 can promote tumorigenesis and may be associated with a poor prognosis in various tumors (7, 8). Interestingly, King et al. (1996) and Shiina et al. (1996) have showed an association of Bcl-2 expression with lower tumor grade and a clinically less aggressive phenotype in TCC patients (9, 10). However, others have shown that expression is more frequent in higher stage and higher grade tumors, which has resulted in higher disease recurrence and progression, as well as shortened survival (11–14).

In contrast to Bcl-2, data on the expression of executive apoptotic molecules in TCC of the urinary bladder are rather scarce. Caspase-3 (CPP32) is a member of the cysteine proteases family that plays a main role during apoptosis (15, 16). It belongs to effector caspases, that are activated by initiator caspases or the mitochondrial pathway (over cytochrome c release into the cytosol) (17). CPP32 is thought to be responsible for the actual destruction of the cell by cleaving multiple structural and repair proteins, since it is the most downstream enzyme in the apoptosis-inducing protease pathway (18, 19). The Bcl-2 family of intracellular proteins are central regulators of caspase activation. On the other hand, CPP32 is able to reverse the function of Bcl-2 by cleaving it to a truncated, proapoptotic form (18). Results concerning the correlation of CPP32 expression and tumor stage or grade in TCC, are so far insufficient (18). Furthermore, to the best of our knowledge, the expression of CPP32 and Bcl-2 protein has not been investigated simultaneously with respect to TCC invasiveness.

To get more insight into the roles of caspase-3, the caspase best correlated with initiation of apoptosis, and Bcl-2 antiapoptotic protein in the malignant phenotype of TCC of the urinary bladder, in this study we determined the expression of these two molecules in patients with non-invasive and muscle-invasive bladder tumors. Levels of expression were correlated with tumor stage and grade.

Materials and Methods

Materials

Tumor samples were obtained from 84 patients (20 female and 64 male) with TCC of the urinary bladder, who underwent transurethral resection, partial or radical cystectomy. The average age of patients was 64.89 ± 10.02 years. None of the patients had a history of chemotherapy. All patients gave written informed consent to participate in the study, which was approved by the Institutional Review Board of the Faculty of Medicine, University of Belgrade.

Sample preparation

Specimens of tumor tissue were taken in the operating theatre in the presence of a clinical pathologist, who performed the histopathologic examination. The TCC samples were staged according to the International Union against Cancer and graded 1 to 3, according to the World Health Organization criteria. The tissues were routinely processed with 10% buffered formalin fixation and paraffin embedding. The H-E stained slides were retrieved and appropriate blocks were selected for immunohistochemical staining.

Immunohistochemical staining for Bcl-2 protein and CPP32

For immunohistochemical staining, with antibodies for both Bcl-2 and CPP32, 4 mm thick serial tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was eliminated by incubation with 3% H_2O_2 in 10% methanol for 5 minutes, at room temperature. The Bcl-2 antigen was retrieved by microwaving in a 750W microwave oven for 15 minutes, followed by microwaving at 450W for 10 minutes in 0.01 mol/L citrate buffer solution, pH 6.0. The CPP32 antigen was retrieved by microwaving in a 700W microwave oven in a 0.01 mol/L citrate buffer solution, pH 6.0, for 30 minutes. The slides were then incubated with primary antibodies for Bcl-2 (*ready to use*) (*Dako, Glostrup, Germany*) and CPP32 (1:50) (*Dako, Glostrup, Germany*), for 2 hours at 37 °C and for 1 hour at room temperature, respectively. They were then incubated with *Chem-Mate Detection Kit* (*Dako, Glostrup, Germany*). The kit is based on the LSAB (labelled streptavidin-biotin) method and is employed in a three-step procedure: incubation with an optimally diluted primary rabbit/mouse antibody, incubation with biotinylated secondary antibodies and with streptavidin peroxidase. The reaction is visualized by 3-amino-9-ethylcarbazole (AEC) chromogen. The sections were counterstained with hematoxylin (*Mayer)*. In all cases, human tonsil tissue was used as the Bcl-2 positive control and CPP32-positive normal cells within the lymph node strongly served as positive control.

Interpretation for immunostaining

The immunostaining, for both Bcl-2 and CPP32, was evaluated on two occasions by one observer, who was unaware of the other data.

The analysis of immunostaining results for Bcl-2 was performed by light microscopy observation, for the presence or absence of positivelly stained tumor cells.

Semiquantitative analysis for CPP32 immunostaining was also performed by light microscopy observation, according to the following, statistically appropriate, cut-off points: (–) or negative status, when less than 10% of the epithelial cells were stained; (+) or low-positive status, when more than 10% and less than 50% of the cells were stained; (++) or medium-positive status, when 50–75% of the cells were stained and (+++) or high-positive status, when more than 75% of epithelial cells were stained.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 11, SPSS Inc, Chicago, IL) software. Analysis of data was accomplished using Kruskall-Wallis or Mann-Whitney tests for non-parametric data. Comparison of Bcl-2 protein and CPP32 immunostaining was made by the chi-squared test. Correlation between expression of examined proteins was tested using Spearman's test. $P<0.05$ was considered to be statistically significant.

Results

Tumor samples were obtained from 84 patients with TCC of the urinary bladder. Out of the total number, 64 patients (76%) were men. With regard to the level of infiltration, of the 84 TCCs, 41 were pT1, 33 were pT2, 5 were pT3 and 5 were pT4. Grading was as follows: 31 were G1, 20 were G2 and 33 were G3. Therefore, staging showed a superficial growth pattern in 41 patient (49%), while tumor samples from other 43 patients (51%) showed invasive chacteristics.

Expression of Bcl-2 protein in TCC of the urinary bladder

Expression of Bcl-2 antiapoptotic protein was positive in 40 out of 84 (48%) patients with TCC of the urinary bladder. Distribution of frequencies of Bcl-2 positive and Bcl-2 negative patients, according to tumor grade and tumor stage, is presented in *Table I*. Representative Bcl-2 negative and Bcl-2 positive staining is presented in *Figure 1A* and *1B*, respectively. As presented in *Table I*, the frequency of Bcl-2 negative patients decreased with tumor grade, while the frequency of Bcl-2 positive patients increased with tumor grade. The observed change in Bcl-2 expression in regard to tumor grade is statisticaly significant ($p=0.032$). When related to tumor stage, the expression of Bcl-2 antiapoptotic protein changed in a very similar manner. Namely, there is a statisticaly significant association of Bcl-2 protein expression and tumor stage ($p=0.007$). Regarding the growth pattern, there was a statisticaly significant difference in the Bcl-2 protein expression in superficial compared to invasive transitional cell carcinoma of the urinary bladder ($p=0.005$), with the frequency of Bcl-2 positive patients being higher in muscle-invasive TCC.

Expression of CPP32 in TCC of the urinary bladder

Expression of CPP32, the most downstream apoptosis-inducing enzyme, was positive in 67 out of 84 (80%) patients with TCC of the urinary bladder. Low-positive status was observed in 15/84 (18%), medium-positive status in 20/84 (24%) and high-positive status in 32/84 (38%) patients. Distribution of frequencies of CPP32 negative and CPP32 low-, medium- and high- positive patients, according to tumor grade and tumor stage, is presented in *Table I*. Representative high-positive, medium-positive and

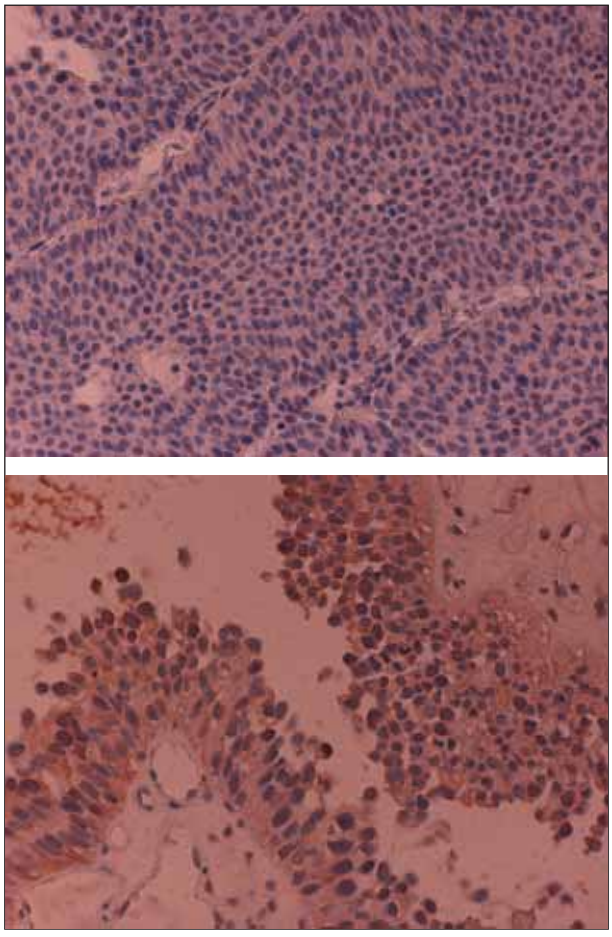

Figure 1 Expression of Bcl-2 protein in TCC.

Peroxidase immunostaining for Bcl-2 protein in transitional cell carcinoma. **A**. Bcl-2 negative staining, grade I. Original magnification x 40. **B**. Bcl-2 positive staining, grade III. Original magnification x 40.

CPP32 negative staining is presented in *Figure 2A*, 2B and 2C, respectively. Regarding tumor grading, the frequency of CPP32 negative patients increased with tumor grade. At the same time, the frequency of CPP32 high-positive patients decreased with tumor grade. Still, no statistically significant association was found between the CPP32 expression and tumor grade (p=0.140). A similar pattern could be observed regardning tumor staging, with the exception of T4 staged tumor samples. Namely, the frequency of CPP32 negative patients increased with tumor stage and frequency of high-positive patients decreased with tumor stage, but only in T1–T3 tumor samples. Nevertheless, there was a statistically significant association between the CPP32 expression and tumor stage (p = 0.043). Furthermore, when classified according to the growth pattern, there was a statisticaly signifficant difference in CPP32 expression between superficial and invasive transitional cell carcinoma of the urinary bladder (p=0.032), with the frequency of CPP32 negative patients being higher and CPP32 high-positive patients being lower in muscle-invasive TCC.

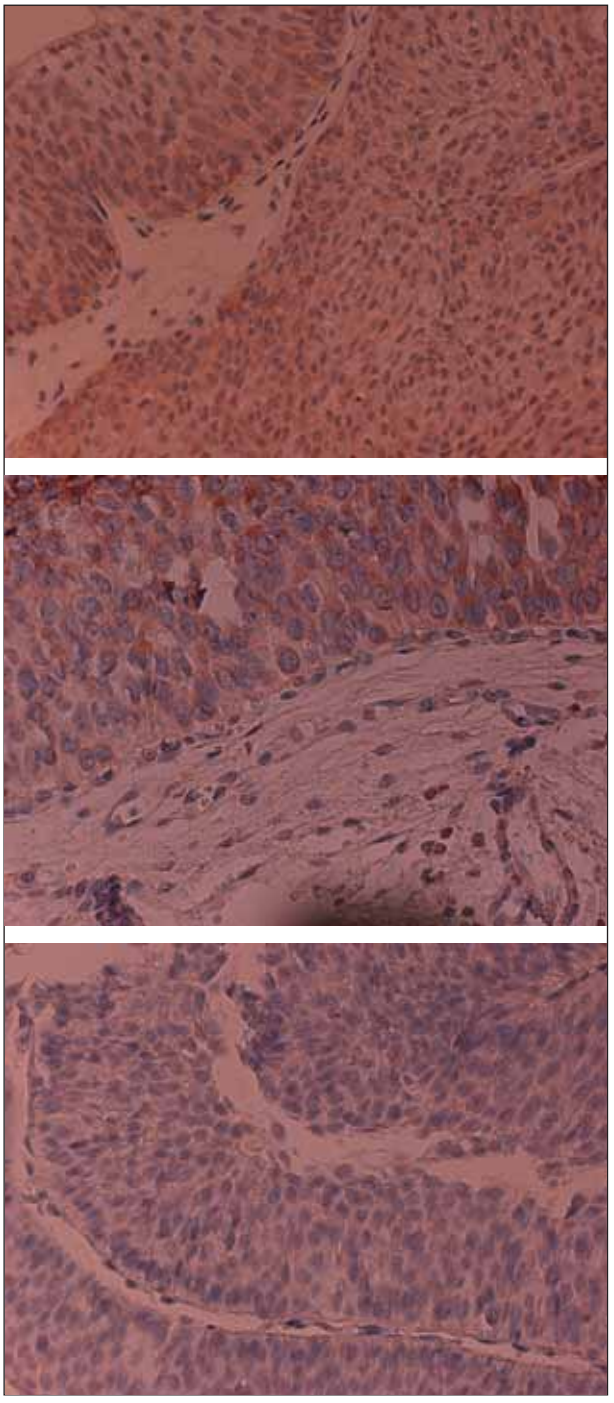

Figure 2 Expression of CPP32 in TCC.

Peroxidase immunostaining for CPP32 in transitional cell carcinoma. **A.** CPP32 high-positive status, grade I. Original magnification x 40. **B**. CPP32 medium-positive status, grade II. Original magnification x 40. **C.** CPP32 negative status, grade III. Original magnification x 40.

Table I Expression of Bcl-2 and CPP32 in TCC of the urinary bladder.

	Bcl-2, n (%)		CPP32, n (%)			
	Negative	Positive	Negative	Positive		
				(+)	(++)	(+++)
Tumor grade (G)						
GI	22 (71)	9 (29)	3 (10)	7 (23)	6 (19)	15 (48)
GII	9 (45)	11 (55)	5 (25)	2 (10)	4 (20)	9 (45)
GIII	13 (39)	20 (61)	9 (28)	6 (18)	10 (30)	8 (24)
Tumor stage (T)						
T1	28 (68)	13 (32)	5 (12)	7 (17)	9 (22)	20 (49)
T2	15 (45)	18 (55)	8 (25)	6 (18)	9 (27)	10 (30)
T3	1 (20)	4 (80)	3 (60)	1 (20)	1 (20)	0(0)
T4	0 (0)	5 (100)	1 (20)	1 (20)	1 (20)	2 (40)

Table II Relationship between Bcl-2 and CPP32 expression in TCC of the urinary bladder.

Bcl-2, n (%)	CPP32, n (%)				
	Negative	Positive			Total
		+	++	+++	
Positive	9 (11)	7 (8.0)	9 (11)	15 (18)	40 (48)
Negative	8 (9.5)	8 (9.5)	11 (13)	17 (20)	44 (52)
Total	17 (20)	15 (18)	20 (24)	32 (38)	84 (100)

χ^2 P = 0.040

Relationship between Bcl-2 protein and CPP32 expression in TCC of the urinary bladder

The positive rate of Bcl-2 protein was lower than that of CPP32 in TCC of the urinary bladder. A statistically significant association was observed between the expression of Bcl-2 negative and CPP32 positive patients (p=0.040). Namely, the highest number of tumor samples that were Bcl-2 negative at the same time showed CPP32 high–positive status (*Table II*). Still, no statistically significant correlation was found when Bcl-2 positive/negative samples were correlated with the CPP32 positive/negative tumor samples (r=–0.054).

Discussion

Mounting evidence exists that alterations in caspases participate in tumor development (5, 20, 21). However, CPP32 expression has not been directly linked to the apoptotic index of malignant cells in TCC (18). Furthermore, there are rather conflicting data concerning the association of CPP32 expression and tumor grade and stage (18, 22). Our results on CPP32 expression in TCC of the urinary bladder have shown a significant association between CPP32 expression and tumor stage. Interestingly, the association, which clearly exists in T1–T3 staged tumors, is lacking in T4 staged tumors. That might be explained by a small number of T4 samples we were able to collect. Contrary to tumor stage, a significant association between CPP32 expression and tumor grade is lacking in this study. The results of Shen et al. (2004) have shown a correlation between CPP32 expression and tumor grade, but no correlation with tumor stage, while Giannopoulou et al. (2002) have found no association between CPP32 expression with neither tumor stage nor tumor grade in TCC of the urinary bladder (18, 22). Remarkably, none of these studies investigated the CPP32 expression in TCC in the context of tumor invasiveness. However, our results on CPP32 expression, regarding the growth pattern, indicate a significant difference in the CPP32 expression between superficial and muscle-invasive TCC. Namely, a dominant finding is the high frequency of CPP32 negativity and low frequency of CPP32

high-positivity in muscle-invasive TCC. The detected change in CPP32 expression, which appears as the tumor invades the muscle in urinary bladder, might possibly confirm the assumption that alterations in caspase expression participate in tumor development.

In contrast to CPP32, there are much more data on the expression of Bcl-2 protein in TCC, although the data are also conflicting. The antiapoptotic Bcl-2 protein belongs to the family of proteins that are »decision-makers«. When overexpressed, Bcl-2 disrupts the normal regulation of pro- and antiapoptotic factors and tips the balance to an antiapoptotic stand (23). There are literature data indicating that healthy bladder tissue has low Bcl-2 expression (24). Increased Bcl-2 expression in low grade tumors of the urinary bladder supports the hypothesis that up-regulated Bcl-2, and the consequent block of apoptotic pathways, may represent first step in bladder carcinogenesis (25). Eissa and Seada (1998) have shown that poorly differentiated tumors have higher Bcl-2 expression than lower grade tumors (24). Many researchers agree that Bcl-2 overexpression should be associated with tumor grade and stage, but only few have found a significant association between these parametres (6, 8, 22). Our results on a significant correlation between Bcl-2 expression and tumor grade are in concordance with a results of Li et al. (1998), who have shown that Bcl-2 oncoprotein is expressed in a high portion of high-grade TCC, but is often absent in superficial or low-grade carcinoma (26). We also found a significant association with tumor stage in all samples, including T4 staged tumors. The most important change in Bcl-2 expression in TCC, which was detected in our study, is the shift in ratio between Bcl-2 negative and Bcl-2 positive patients that exists between superficial and muscle-invasive TCC of the urinary bladder. This finding is in agreement with the proposal that Bcl-2 positivity is a marker of disease more likely to progress and of poor prognosis (8). Thus, elucidation of the expression of apoptotic molecules in TCC may affect not only the understanding of the progression and behavior of invasive tumors, but also cancer therapy in TCC patients. As recently suggested, intravesical and systemic chemotherapy in bladder cancer are rather limited in their efficacy due to the inability of chemotherapeutic agents, in the treatment of advanced TCC, to induce apoptosis in bladder tumor cells (1, 27). Since recent data also indicate that new treatment options are necessary for superficial bladder cancer, owing to the high recurrence rate after conventional treatment (25), it might be hypothesized that drugs that target apoptotic molecules may improve cancer treatment. In that context, Hussain and James (2005) suggested that, since Bcl-2 positivity predicts poor survival with chemotherapy, drugs which target Bcl-2 may improve clinical outcome in patients with TCC of the urinary bladder (1). Still, before we can see the impact of these new prognostic markers and their influence on cancer treatment and improved patient care, they must be evaluated critically in the subgroups of muscle-invasive tumors.

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