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RESEARCH ARTICLE

Role of Trop2, Cyclin D1 and FOXP3 in bladder carcinoma in Egyptian patients: An immunohistochemical study

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ABSTRACT

Background: In Egypt, Urinary bladder carcinoma is a common malignancy accounting for 14.3% of total malignancies in both sexes with a 3:1 M: F ratio. To reduce bladder cancer morbidity and mortality, identification of tumor markers specific enough for prognosis and can serve as an effective anti-cancer target is urgent. The purpose of this study is to evaluate the role of Trop2, Cyclin D1, FOXP3 and their relationship with the established clinicopathological parameters and overall survival of bladder cancer patients. **Methods**: Using the standard immunohistochemical technique in 80 primary bladder carcinomas and 20 specimens as non-neoplastic groups. The malignant group included 50 cases of muscle-invasive and 30 cases of non-muscle invasive bladder cancer. **Results**: significant association of overexpressed Trop2 and FOXP3 with high grade, advanced stage, lymph node involvement, and high mitotic count. On the other hand, Cyclin D1 displayed a favorable prognostic impact and an inverse relation with Trop2 and FOXP3. A direct correlation between both FOXP3 expression in malignant cells and peritumoral TIL FOXP3+ expression was displayed. Trop2, Cyclin D1, FOXP3 expression didn't affect the overall survival of the studied sample. **Conclusions**: The inverse relation between Cyclin D1 and Trop2 proposes the consumption of Cyclin D1 by Trop2 as a ligand in the urinary bladder carcinogenesis. A synergistic role and a cross-talk between TIL FOXP3+ and tumoral FOXP3 + cells are anticipated. Trop2 and FOXP3 could be a promising potential biomarkers for identifying patients with poor prognostic factors in bladder cancer serving as potential targets for cancer therapy

Keywords: Trop2, Cyclin D1, FOXP3, BC, Immunohistochemistry

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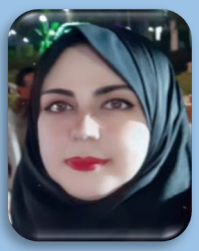

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INTRODUCTION

In Egypt, Urinary bladder carcinoma is a common malignancy accounting for 14.3% of total malignancies in both sexes with 3:1 male to female ratio. It comprises 88.3% of the total urinary system tumors according to the National cancer institute registry 2016 (Ibrahim et al., 2014). The expected new cases are about 10.709 by 2020, 12.762 by 2025 and 28.337 by 2050 (Helal et al., 2015, Kyritsi et al., 2018).

To reduce bladder cancer morbidity and mortality, there is an urgent need to identify novel tumor markers which are specific enough for prognosis and can serve as effective anticancer targets (Tang et al., 2019; Heabah & Bedeer, 2021). Trop2 is a transmembrane glycoprotein encoded by the Tacstd2 gene (Fong et al., 2008a), which has been actively studied as a prognostic marker and an attractive immunotherapeutic target in human cancer treatment (Guerra et al., 2013). Trop2 has several ligands, including claudin-1, claudin-7, cyclin D1, and potentially IGF-1, as for cyclin D1 is a protein encoded by CCND1 gene and it is required for progression through the G1 phase of the cell cycle (Baldin et al., 1993). Trop2 forms an oncogenic fusion protein with cyclin D1 (Huang et al., 2005). This chimera is expressed by human tumors differentially (Cubas et al., 2009). FOXP3 is a forkhead box transcription factor containing a DNA-binding domain (Lopes et al., 2006), it is known as the most specific marker of the regulatory T lymphocytes (Tregs) (Fontenot et al., 2003). FOXP3 plays a crucial role in the development

and function of Tregs, it is constitutively expressed in the nucleus of human Tregs (Martin et al., 2010).

In the scope of further understanding bladder carcinogenesis and the prognostic and predictive factors affecting it, emanates the aim of this study in investigating the role of Trop2, Cyclin D1 and FOXP3 in bladder carcinoma and correlate their expression with the available clinicopathological parameters and overall survival.

METHODS

This retrospective study included 80 primary bladder carcinoma and 20 non-neoplastic bladder specimens. The bladder carcinoma cases were received as radical cystectomy specimens (50 cases of muscle-invasive bladder cancer) and Transurethral Resection of Bladder Tumor (TURBT) (30 cases of non-muscle invasive bladder cancer). The 20 cases of the control group were received as cystoscopic biopsies. The cases were retrieved from the archives of Pathology Department, Faculty of Medicine, Menoufia University spanning the period between January 2017 and December 2019.

Clinical data of the studied groups: Clinical data regarding the bladder carcinoma cases were obtained from patients' medical records and documented in Table1.

Histopathological Assessment: Hematoxylin and eosin (H&E) stained sections were evaluated for the following; Histological type according to WHO classification, 2016. Tumor grading was done according to WHO/ISUP grading criteria (Moch et al., 2016). Mitotic and apoptotic counts were counted semi-quantitatively in ten randomly selected high power fields (Chauhanet al., 2016) Depth of invasion and staging of the tumor were assessed according to TNM staging system/American Joint Committee on Cancer (AJCC) Staging manual 8th edition. According to TNM classification for the stage; the malignant tumors were classified histologically as non-muscle-invasive urinary bladder carcinoma (NMIUBC) (stage pTa and pT1) or muscle-invasive urinary bladder carcinoma (MIUBC) (stage pT2, pT3 and pT4) (Boustead et al., 2014).

Immunohistochemistry: The method used for immunostaining was streptavidin-biotin amplified system. Sections cut from the paraffin-embedded blocks were stained with Anti-Trop2 (cat# 241308, abbexa, UK) purified rabbit poyclonal antibody was received as 0.1 ml conc. and diluted by phosphate buffer saline (PBS) in a dilution of 1:100. Anti-Cyclin D1 (cat# RM-9104-R7, Thermo Fisher Scientific, USA) rabbit polyclonal antibody was received as a ready to use 7 ml vial. Anti-FOXP3 (cat # ARP32743, Aviva Systems Biology, USA) rabbit polyclonal antibody was received as 0.1 ml. conc. and diluted by PBS in a dilution of 1:100. Tissue sections prepared from, normal skin as a positive control for Trop2 (Avellini et al., 2017), from normal human tonsil for Cyclin D1 (Amer and Eid 2019) and spleen for FOXP3 (18). Negative control slides were also included in each run by omitting the primary antibody.

Cytoplasmic and/or membranous staining in any number of tumor cells for Trop2 were required to assign the positivity (Avellini et al., 2017). Nuclear staining in any number of cells for Cyclin D1 was required to assign the positivity (Amer and Eid, 2019) FOXP3 was assessed in malignant epithelial tissues and the intra-tumoral and peri-tumoral infiltrating lymphocytes, cytoplasmic and nuclear staining in any number of tumor cells and tumor infiltrating lymphocytes whether (peri-tumoral or intratumoral) respectively were required to assign the positivity (Winerdal et al., 2011).

For all studied markers (Trop2, Cyclin D1 and FOXP3), H-score system (Histochemical score): was applied to evaluate the studied section according to (Smyth et al., 2007). H score Formula = strong intensity (3) x percentage + moderate intensity (2) x percentage + mild intensity (1) x percentage. H-score ranged from 0 to 300 and it was assessed as mean±SD, median and range.

Immunoreactivity score (IRS): was calculated depending on both intensity scoring and extent of positivity which was graded according to the German semi-quantitative scoring system as following (Godlewski et al., 2018). Intensity scoring (IS): 0= No staining, 1= Weak staining, 2= Moderate staining, 3= Strong staining. Extent of positivity was scored by percent of positive cells

as , 0= 0% , 1= 1–10% , 2= 11–50%, 3= 51–80%, 4= >80% . The immunoreactive score (IRS) was determined by multiplying intensity scoring by the extent of positivity (0 to 12). The cases were categorized into low and high expression scores as, Low expression Score: If the score is less than 6. High expression score: If the score is more than or equal to 6.

Statistical Analysis: The statistical analysis was conducted using SPSS "statistical package for the social science" program for windows, version 22.0 (SPSS INC., Chicago, Illinois, USA). Contingency tables were analyzed with descriptive statistics [Arithmetic mean ($\bar{x}$), Standard deviation (SD), Percentage (%), Median and Range] and analytic statistics[Chi-square test (X^2- test), Mann-Whitney Z test (Z test), Kruskal-Wallis test (K test), Fisher's exact (FE)]. Overall survival (OS) was analyzed using the Kaplan–Meier method, and differences were examined using log-rank tests. Cox's proportional hazard regression test was used to estimate univariate and multivariate hazard ratios for prognosis. P values of ≤ 0.05 were considered statistically significant (Dawson and Trapp 2001).

RESULTS

Clinicopathologic characteristics: of primary bladder carcinoma cases (80 cases) are summarized in Table 1, as for the clinical data of the non-neoplastic group (20) their age ranged from 30 to 77 years with a mean±SD of 56.8±10.9 years with a median age of 55 years, 18 cases (90%) were male and 2 cases (10%) were female (Table 1).

Comparison between malignant and control groups regarding Trop2, Cyclin D1 and FOXP3 immunohistochemical profile (Table 2): The expression of the studied Trop2, Cyclin D1 and FOXP3 between both non-neoplastic and UBC groups failed to reach a statistical significance. Meanwhile, membranous Trop2 was exclusively associated with the malignant group (p=0.005) while patchy cytoplasmic FOXP3 was in favor of non-neoplastic group (p=0.011 and 0.001)

Relationship between Trop2, Cyclin D1 and FOXP3 IRS score and clinicopathological parameters in primary bladder carcinoma cases (Tables 3-6): Trop2 high immunoreactive score (IRS) was statistically in favor of high grade (P=0 .017), advanced stage (P=0.001), presence of lymph node involvement (P=0.001), LVI (P= 0.001), PNI (P= 0.005) and high mitotic count (P= 0.001) (Table 3 and Figure 1). Cyclin D1 high Immunoreactive score (IRS) was correlated with an early-stage group (P=0.001) and low mitotic count (P=0.007). Moreover, all high IRS Cyclin D1 cases (20/20) displayed an absence of bilharzial infestation (P=0.001) (Table 4 and Figure 2). Regarding tumoral FOXP3 immunoreactive score (IRS), high FOXP3 IRS was significantly in favor of advanced stage (p=0.003), lymph node metastasis (p=0.025), LVI and high mitotic count (P=0.008) (Table 5 and Figure 3). Regarding the peritumoral FOXP3 expression, positive FOXP3 was statistically in favor of inflammatory stromal reaction (P=0.001) and advanced stage, for 21 out of 33 TIL FOXP3 positive cases were observed in the advanced stage bladder cancer (P=0.001) (Table 6). A significant direct relationship between tumoral FOXP3 H-Score and peritumoral positively stained FOXP3+ TIL H-score was observed in the current study (p=0.001) (Table 7).

Correlation between Cyclin D1 H-score, Trop2 and tumoral FOXP3 H-scores in malignant cases: There was a significant inverse relationship between Cyclin D1 H-score and both Trop2 and tumoral FOXP3 H-scores (P=0.001 and 0.005 respectively) (Table 7).

Survival analysis: Univariate analysis of overall survival showed the bad prognostic impact of advanced pathological T stage (P=0.003), nodal invasion (P=0.006) and bilharzial infestation (P=0.044) on patient's outcome (Table not shown). None of the studied primary antibodies Trop2, Cyclin D1, tumoral and peritumoral FOXP3 revealed a significant impact on the overall survival of the patients included in the study (Table not shown**)**

DISCUSSION

In the current study Trop2, was expressed in 85% of the non-neoplastic urothelium and in 97.5% of the malignant group (P>0.05) in agreement with (Zhang et al., 2017; Ohmachi et al., 2006; Bignotti et al., 2010; Stepan et al., 2011).

Table 1. Clinicopathological data of the studied bladder carcinoma cases (no.=80)

Variables		Carcinoma Group (No=80)	
Age(years)	Mean±SD	63.5±10.3	
	Median	65	
	Range	26 – 96	
Gender		No	%
	Male	66	82.5
	Female	14	17.5
Tumor Size (50 cases)	Mean±SD	4.7±1.2	
	Range	4.7	
	Median	3-9	
WHO Histologic Type (2016)		NO	%
	Infiltrating UC	76	95
	Noninvasive papillary UC	4	5
Grading	Low	18	22.5
	High	62	77.5
Muscularis propria invasion	NMUIBC	30	37.5
	MIUBC	50	62.5
Tumor stage	Ta	4	5
	T1	26	32.5
	T2	18	22.5
	T3	28	35
	T4	4	5
Stage grouping	Early	30	37.5
	Advanced	50	62.5
Lymph node stage (no=50)	N0	20	40
	N1	11	22
	N2	19	38
Bilharziasis	Present	24	30
	Absent	56	70
LVI	Present	20	25
	Absent	60	75
PNI	Present	10	12.5
	Absent	70	87.5
Necrosis	Present	28	35
	Absent	52	65
Stromal reaction	Desmoplastic	39	47.5
	Inflammatory	29	36.3
	Both	13	16.2
Mitosis	Mean±SD	5.8±2.6	
	Range	2 – 15	
	Median	5	
Apoptosis	Mean±SD	15.0±6.5	
	Range	3 – 31	
	Median	14	

SD: Standard deviation, No: number, UC: Urothelial carcinoma

Transcription factors are known to be involved in cancer cell progression, such as WT1 regulate Trop2 transcription (Zaman et al., 2019), in addition, overexpression of Trop2 may be due to Trop2's intrinsic regulatory effects on cancer cell growth, invasion, and proliferation (Shyartsur and Bonavida, 2015). Overexpression of Trop2 naturally leads to tumor progression as a key driver of cancer growth (Guerra et al., 2013). On the other hand, Trop2 was found to be overexpressed across normal tissues in animal models, including the bladder, uterus, kidney, lung, and skin (Stepan et al., 2011).

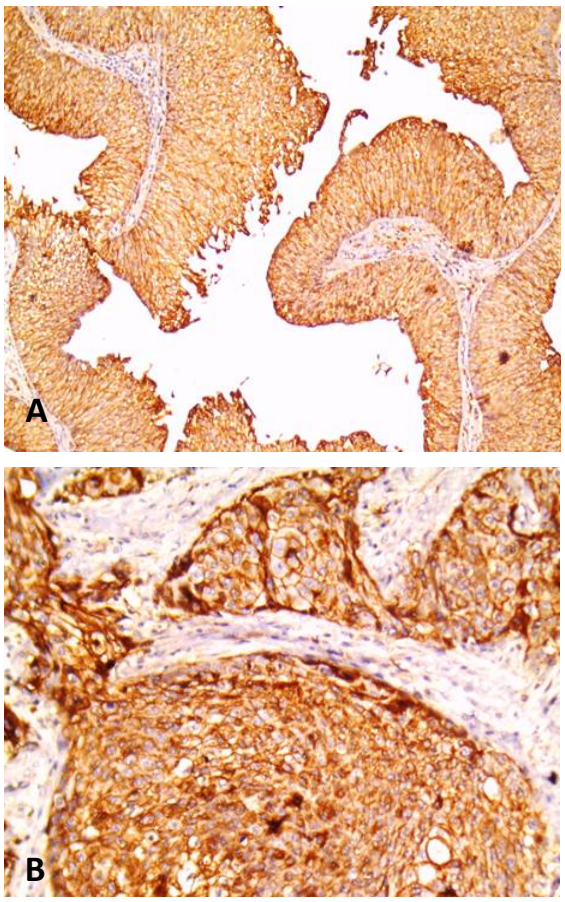

Figure 1. A): A case of non invasive papillary urothelial carcinoma showed strong membranocytoplasmic immunoreactivity of TROP2 (Immunoperoxidasex100). B): Strong diffuse membranocytoplasmic immunoreactivity of TROP2 in a case of muscle invasive urinary bladder carcinoma (Immunoperoxidasex200).

The expression of Trop2 in normal tissues may play an important role in normal tissue homeostasis. EpCAM, the Trop2 paralog, is thought to function as an epithelial cell adhesion molecule (Litvinov et al., 1997; Balzar et al., 2001). Trop2 shares a conserved cysteine-rich region in its extracellular domain that is required for EpCAM-mediated adhesions (Balzar et al., 2001). Trop2 high immunoreactive score (IRS) was significantly associated with poor prognostic factors as high grade, advanced stage, presence of lymph node involvement, LVI, PNI and high mitotic count. These results are in agreement with (Zhang et al., 2017) who demonstrated a significant association of overexpressed Trop2 with high tumor grade, advanced stage, and recurrence in UBC, not only in bladder cancer but also in gastric carcinoma.

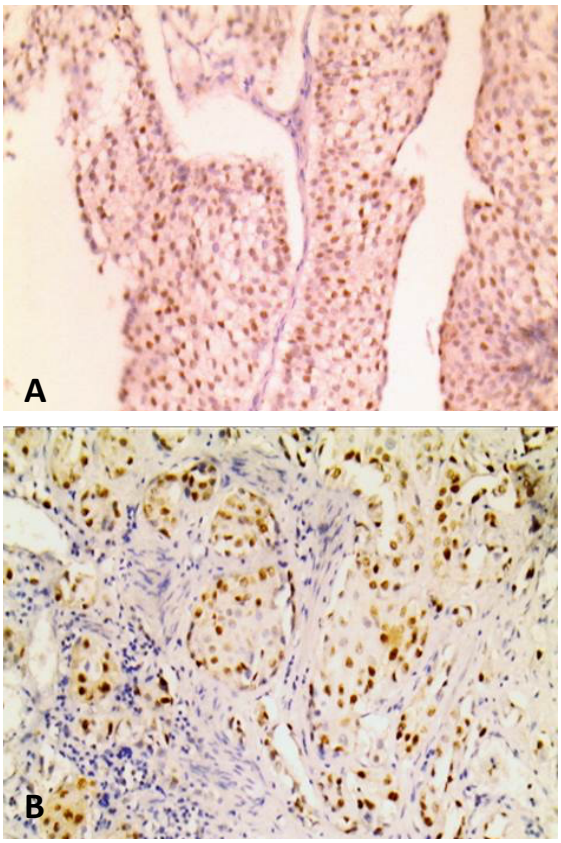

Figure 2. A): Moderate to strong nuclear Cyclin D1 immunoreactivity in a case of Non-invasive papillary low-grade urothelial carcinoma (Immunoperoxidase x 200). B): Moderate to strong nuclear Cyclin D1 immunoreactivity in a case of muscle-invasive urinary bladder carcinoma (Immunoperoxidase x200).

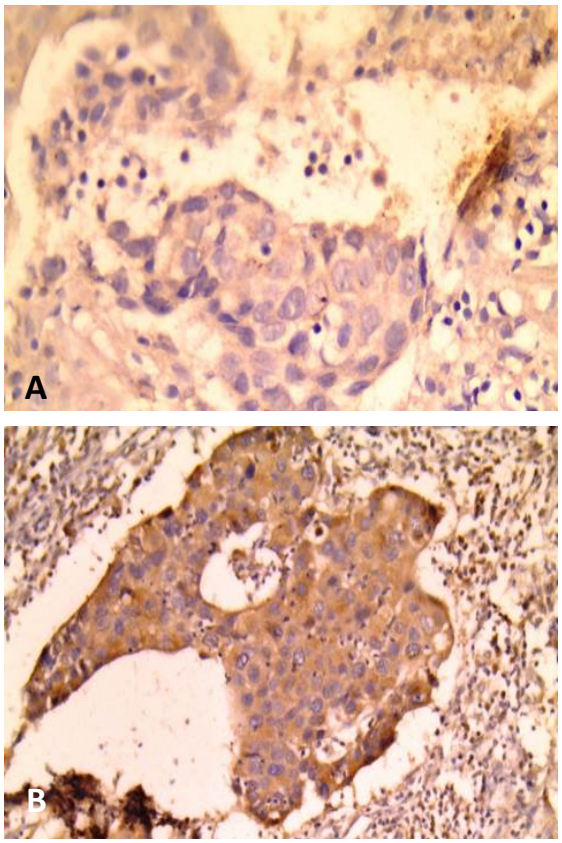

Figure 3. A): Mild cytoplasmic epithelial FOXP3 immunoreactivity with negative peritumoral lymphocytes in a case of non-muscle invasive urinary bladder (Immunoperoxidasex400). B): Moderate to strong cytoplasmic epithelial FOXP3 with moderate scattered strong nuclear peritumoral lymphocytes FOXP3 immunoreactivity in a case of muscle-invasive urinary bladder carcinoma (Immunoperoxidasex200).

Studies such as (Li et al., 2016; Li et al., 2017; Tang et al., 2019) stated that overexpression of Trop2 enhances the proliferation, migration, and invasion of malignant cells in lung cancer, gall bladder cancer and oral squamous cell carcinoma (OSCC) respectively, while downregulation of Trop2 triggered apoptosis and impaired proliferation. Activation and regulation of ERK pathway in cervical cancer cells and regulation of PI3K/AKT pathway inducing EMT in gall bladder cancer by Trop2 was stated by studies done by (Li et al., 2013; Li et al., 2017). Moreover, studies done by (Zhang et al., 2014; Redlich et al., 2018) found that loss of Trop2 leads to autocrine activation of the EGFR family member ErbB3 through neuregulin-1 in the mesenchymal subtype of head and neck squamous cell cancer (HNSCC) and induced sensitivity to anti-ErbB3 antibodies, leading to reduced proliferation and tumorigenic growth in HNSCC cells.

Regarding Cyclin D1, it was expressed in 85% of the non-neoplastic urothelium and in 76.2% of the malignant group (P>0.05), in the current study. Our results are in congruity with Khabaz et al., 2016 who reported Cyclin D1 immunoreactivity similarly frequent in bladder tumors (51.6%) and normal tissue of bladder (50%). A study done in 2007 by (Shariat et al., 2007) agreeing, reported uniformly intense expression of Cyclin D1in the non-neoplastic group. However other studies (Kopparapu et al., 2013) reported higher Cyclin D1 protein expression in UBC and in endometrial carcinoma *(*Yan et al., 2017*)* compared to the adjacent normal tissue. Meanwhile, other studies (Takagi et al., 2000; Mhawech et al., 2004; Fristrup et al., 2014) reported complete absence of Cyclin D1 in normal urothelium and in other tissues as colonic and gastric mucosa (Bahnassy et al., 2004; Gao et al., 2004; Shan et al., 2017) expressed in the carcinoma group.

Table 2. Comparison between the non- neoplastic group and UBC group regarding the immunohistochemical profile of the studied TROP2, Cyclin D1 and FOXP3

Variables		Non neoplastic group (No=20)		Malignant group (No=80)		Test of significance	P-value
		No	(%)	No	(%)		
Trop2 positivity	Positive Negative	17 3	85 15	78 2	97.5 2.5	FE= 5.6	0.056
		No=17		No=78			
Percentage	Mean±SD Range Median	64.1±15.9 30– 90 70		72.0±19.2 20 – 100 77.5		FE=1.7	0.081
Trop2 Subcellular localization	Membranous Membranocytoplasmic	0 17	0 100	52 26	66.7 33.3	FE=7.8	0.005*
Trop2 predominant intensity	Mild Moderate Strong	0 4 13	0 23.5 76.5	10 27 41	12.8 34.6 52.6	FE= 4.1	0.128
Trop2 pattern of staining	Patchy Diffuse	5 12	29.4 70.6	19 59	24.4 75.6	FE= 0.189	0.759
Trop2 IRS	Low High	6 11	35.3 64.7	31 47	39.7 60.3	X^2= 0.116	0.733
Trop2 H Score	Mean±SD Median Range	174.1±56.3 50 – 240 180		182.5±73.9 20 – 300 190		FE= 0.696	0.487
Cyclin D1 expression	Positive Negative	17 3	85 15	61 19	76.2 23.8	FE= 0.714	0.551
		No=17		No=61			
Percentage	Mean± SD Range Median	39.4±21.5 10 – 80 40		48.4±21.6 10 – 90 50		U= 1.5	0.123
Predominant intensity		NO	%	NO	%	FE= 1.7	0.425
	Mild Moderate Strong	2 10 5	11.8 58.8 29.4	10 25 26	16.4 41 42.6		
Cyclin D1 IRS	Low High	15 2	88.2 11.8	41 20	67.2 32.8	FE=2.9	0.129
Distribution	Patchy Diffuse	11 6	64.7 35.3	43 18	70.5 29.5	x^2=0.209	0.648
Cyclin D1 H.Score	Mean± SD Range Median	88.5±58.1 20 – 220 80		111.1±59.4 10 – 240 120		U=1.4	0.123
FOXP3 Expression	negative positive	3 17	15 85	20 60	25 75	FE= 0.903	0.342
FOXP3 Percent	Mean± SD Range Median	42.9±18.2 10– 60 50		40.0±21.7 10– 80 30		U= 0.720	0.471
FOXP3 Distribution	Patchy Diffuse	11 6	64.7 35.3	54 6	90 10	FE= 6.44	0.011*
FOXP3 Intensity	Mild to moderate strong	16 1	94.1 5.9	42 18	70 30	FE= 5.6	0.060
FOXP3 Subcellular localization	cytoplasmic apical cytoplasmic	9 8	52.9 47.1	60 0	100 0	FE= 31.5	0.001*
FOXP3 IRS	Low High	15 2	88.2 11.8	46 14	6.7 23.3	FE= 1.077	0.299
FOXP3 H score	Mean± SD Range Median	69.4±30.7 20 – 120 60		81.3±53.7 10 – 200 70		U= 0.351	0.726

Table 3. Relationship between Trop2 immunoreactive score (IRS) and studied clinico-pathological parameters in malignant cases

		TROP2 IRS				test of significant	P value
		Low (no=31)		High (no=47)			
		NO	%	NO	%		
Gender	Male (no=64)	24	37.5	40	62.5	x^2= 0.749	0.387
	Female (no=14)	7	50	7	50		
age/years	mean ±SD	63.2±12.1		63.4±9.3		U= 0.092	0.927
	range	26 – 96		46– 83			
	Median	65		64			
WHO Histological Type	Infiltrating UC (no=75)	30	40.5	45	59.5	FE= 0.731	0.694
	Noninvasive papillary UC (no=3)	1	33.3	2	66.7		
Grade	Low (no=17)	11	64.7	6	35.3	x2=5.6	0.017*
	High (no=61)	20	32.8	41	67.2		
stage group	Early (no=28)	24	85.7	4	14.3	x2= 38.5	0.001*
	advanced (no=50)	7	14	43	86		
lymph node (no=50)	Absent (no=20)	4	20	16	80	FE= 39.1	0.001*
	present (no=30)	3	10	27	90		
Bilharasis	absent (no=55)	23	41.8	32	58.2	x2= 0.335	0.563
	present (no=23)	8	34.8	15	65.2		
Necrosis	absent (no=50)	19	38	31	62	0.177 (x2)	0.674
	present (no=28)	12	42.9	16	57.1		
LVI	absent (no=58)	30	51.7	28	48.3	x2=13.5	0.001*
	present (no=20)	1	5	19	95		
PNI	absent (no=68)	31	45.6	37	54.4	FE= 7.6	0.005*
	present (no=10)	0	0	10	100		
Stromal reaction	Desmoplastic (no=25)	16	44.4	9	69.2	x2= 0.809	0.667
	inflammatory (no=31)	11	37.9	20	55.6		
	both (no=22)	4	30.8	18	62.1		
Apoptosis	mean ±SD	14.2±6.5		15.7±6.6		U= 1.13	0.258
	range	3 – 30		4 – 31			
	Median	13		15			
Mitosis	mean ±SD	4.8±1.9		6.6±2.7		U= 3.2	0.001*
	range	2– 11		3 – 15			
	Median	5		7			

Table 4. Relationship between Cyclin D1 immunoreactive score (IRS) and the studied clinico-pathological parameters in malignant cases.

Variables		Cyclin D1 IRS				Test of significance	P value
		Low (no=41)		High (no=20)			
		NO	%	NO	%		
Gender	Male (no=50)	35	67.3	15	32.7	FE= 0.977	0.323
	Female (no=11)	6	66.7	5	33.3		
Age/years	mean ±SD	64.0±8.2		61.4±13.1		U= 0.347	0.729
Grade	Low (no=13)	6	46.2	7	53.8	FE=3.2	0.068
	High (no=48)	35	72.9	13	27.1		
stage group	Early (no=27)	11	40.7	16	59.3	FE= 15.4	0.001*
	Advanced (no=34)	30	88.2	4	11.8		
lymph node (No=34)	Absent (no=14)	13	92.6	1	7.4	Was not suitable statistically	
	Present (no=20)	17	85	3	15	due to small sample size (4) of high IRS	
Bilharziasis	absent (no=45)	25	55.6	20	44.4	FE= 0.6	0.001*
	present (no=16)	16	100	0	0		
LVI	Absent (no=47)	28	59.6	19	40.4	FE= 5.6	0.062
	Present (no=14)	13	92.8	1	7.2		
PNI	Absent (no=56)	37	66.1	19	33.9	FE= 0.404	0.525
	Present (no=5)	4	80	1	20		
Necrosis	Absent (no=40)	26	63.4	14	70	FE= 0.256	0.611
	Present (no=21)	15	36.6	6	30		
Apoptosis	mean ±SD	15.5±6.3		14.5±5.8		K= 0.501	0.617
Mitosis	mean ±SD	6.3±2.7		4.5±1.5		K= 2.7	0.007*

Table 5. Relationship between tumoral FOXP3 IRS and the studied clinico-pathological parameters in malignant cases

Variables		FOXP3 IRS				Test of significance	P value
		Low (no=46)		High (no=14)			
Gender		N O	%	NO	%	FE= 0.007	0.932
	Male (no=51)	39	76.5	12	23.5		
	Female (no=9)	7	77.8	2	22.2		
age/years	mean ±SD	62.5±10.8		66.0±10.4		U= 1.2	0.227
	range	26 – 96		46 – 83			
	Median	64.5		65.5			
WHO Histological type	Infiltrating UC (no=59)	45	76.3	14	23.7	FE= 0.310	0.578
	Noninvasive papillary UC (no=1)	1	100	0	0		
Grade	low (no=12)	10	83.3	2	16.7	FE= 0.373	0.542
	high (no=48)	36	75	12	25		
stage group	early (no=19)	19	100	0	0	FE= 8.5	0.003*
	advanced (no=41)	27	65.9	14	34.1		
lymph node (no=41)	Absent (no=14)	8	57.1	6	42.9	FE= 9.3	0.025*
	Present (no=27)	19	70.4	8	29.6		
Bilharzasis	absent (no=41)	34	82.9	7	17.1	FE= 2.8	0.092
	present (no=19)	12	63.2	7	36.8		
LVI	absent (no=43)	38	88.4	5	11.6	FE= 11.6	0.001*
	present (no=17)	8	41.7	9	52.9		
PNI	absent (no=52)	41	78.8	11	21.2	FE= 1.03	0.309
	present (no=8)	5	62.5	3	37.5		
Necrosis	absent (no=38)	30	78.9	8	21.1	(x^2)= 0.301	0.583
	present (no=22)	16	72.7	6	27.3		
Stromal reaction	Desmoplastic (no=25)	22	88	3	12	FE= 3.6	0.161
	inflammatory (no=26)	17	65.4	9	34.6		
	both (no=9)	7	77.8	2	22.2		
Apoptosis	mean ±SD	14.0±6.5		17.5±8.3		U= 1.22	0.220
	range	3– 31		8 – 31			
	Median	13.5		14.5			
Mitosis	mean ±SD	5.6±2.4		7.6±2.7		U= 2.6	0.008 *
	range	2 – 15		4 – 15			
	Median	5		7			

Cyclin D1 high expression in the current study is associated with the early-stage group (P=0.031), in agreement with several studies (Galmozzi et al., 2006; Lenz et al., 2012; Amer and Eid, 2019; Lee et al., 2010) who stated low level of Cyclin D1 in the advanced stage, poorly differentiated tumors, vascular invasion, as well as lymph node involvement and MIBC.

In the current study, all high IRS Cyclin D1 cases (20/20) displayed absence of bilharzaial infestation (P=0.001) in agreement with a study done by (Zaghloul, 2012) who demonstrated that schistosomiasis associated UBC presented in more advanced stage than schistosomiasis non associated UBC supporting the favorable prognostic impact of Cyclin D1.

The favorable prognostic impact inferred by Cyclin D1 overexpression is attributed to its evidence in the initial stages where cell proliferation is a necessary step, involving no tumor invasion or metastasis as suggested by Guang and Tian (2015) and that low Cyclin D1 expression might be a surrogate of other genetic events in the same cells, which ultimately drives cell growth and leads to worse prognosis (Sud, 1998). Moreover, the phenotype of Cyclin D1 was correlated with the degree of cancer progression and invasiveness.

Altered expression of Cyclin D1 may lead to changes in the biological behavior of transformed cells, for instance, growth, proliferation, invasion and metastasis (Amer and Eid, 2019). The inverse correlation between Cyclin D1expression and poor prognostic parameters was not only reported in urothelial carcinoma; but also among other tumors, as in gastric carcinoma (Feakins et al., 2003), in laryngeal squamous cell carcinoma (Jovanovic et al., 2014) and invasive breast carcinoma (Parvin et al., 2019).

Table 6. Relationship between peritumoral FOXP3 expression and different clinico-pathological parameters in malignant cases

Variables		Peritumoral FOXP3 expression				Test of significance	P value
		positive (no=33)		Negative (no=47)			
		NO	%	NO	%		
Gender	Male (no=66)	28	42.4	38	57.6	(x^2)= 0.215	0.643
	Female (no=14)	5	35.7	9	64.3		
age/years	mean ±SD	62.1±10.0		64.3±10.5		U= 0.387	0.699
	range	26 – 80		42 – 96			
	Median	64		65			
WHO Histological type	Infiltrating UC (no=76)	31	41.3	45	58.7	FE= 0.829	0.661
	Noninvasive papillary UC (no=4)	2	50	2	50		
Grade	Low (no=18)	9	50	9	50	(x^2)= 0.734	0.392
	high (no=62)	24	38.7	38	61.3		
stage group	early (no=30)	12	40	18	60	(x^2)= 0.031	0.001*
	advanced (no=50)	21	42	29	58		
lymph node (no=50)	Absent (no=20)	8	40	12	60	FE= 0.118	0.990
	Present (no=30)	13	43.3	17	65.7		
Bilharasis	absent (no=56)	21	37.5	35	62.5	x^2= 1.1	0.298
	present (no=24)	12	50	12	50		
LVI	absent (no=60)	23	38.3	37	61.7	x^2= 0.842	0.359
	present (no=20)	10	50	10	50		
PNI	absent (no=70)	28	40	42	60	FE=0.361	0.548
	present (no=10)	5	50	5	50		
Necrosis	absent (no=52)	24	46.2	28	53.8	x^2= 1.4	0.225
	present (no=28)	9	32.1	19	67.9		
Stromal reaction	desmoplastic (no=38)	1	2.6	37	97.4	x^2= 44.9	0.001*
	inflammatory (no=29)	23	79.3	6	20.7		
	both (no=13)	9	69.2	4	30.8		
Apoptosis	mean ±SD	14.1±5.9		15.6±6.9		U= 0.656	0.512
	range	3 – 31		4 – 31			
	Median	14		14			
Mitosis	mean ±SD	5.3±2.4		6.2±2.7		U= 1.35	0.176
	range	2– 15		2 – 15			
	Median	5		6			

Table 7. Correlation between peritumoral FOXP3 H-score and tumoral FOXP3 H-score

Markers	FOXP3 in tumor cells	
	r	P- value
FOXP3 immunohistochemistry in peritumoral lymphocytes	**0.421**	**0.001***

Regarding the expression of FOXP3 in the studied groups, FOXP3 was expressed only in the epithelium in the non-neoplastic group and was found to be expressed in both malignant cells and lymphocytes (peritumoral and intratumoral) in cases of the malignant group.

In this current study, FOXP3was expressed in 85% of the normal urothelium and 75% of the tumor cells in the malignant group (P>0.05). This is in contrast to a study done by (Winerdal et al., 2011) and (Zhang t al., 2016) who found that FOXP3 was highly expressed in cancer cells of UBC. Meanwhile, a study done by (Zuo et al., 2007) found that FOXP3 was expressed in normal breast and down-regulated in adjacent mammary cancer, the study clarified that ErbB2and Skp2genes in breast cancer and c-Myc gene in prostate cancer are repressed causing predominant missense mutations in breast and prostate cancer patients suggesting the role of FOXP3 as a tumor suppressor gene (Zuo et al., 2007; Wang et al., 2009).

High FOXP3 IRS , in the current study, is in favor of advanced stage (p=0.003), lymph node metastasis (p=0.025), LVI and high mitotic count (P=0.008). Association of tumoral FOXP3 with poor prognostic factors agrees with a study done by (Zuo et al., 2007) which demonstrated the association of tumoral FOXP3 with lymphatic metastasis, advanced stage and high proliferative index (Ki-67≥14%) in cancer breast.

The prognostic role of FOXP3 in tumor cells has been studied for many years. In vitro, FOXP3 represses the transcription of the HER2, SKP2, MYC, MMP2, and UPA genes and induces the expression of p21 and LATS2. Thus, inhibited cell growth, cell migration and cell invasion have been observed in cell lines derived from breast, prostate and ovarian cancers that overexpress FOXP3 (Peng et al., 2019, Nishikawa & Sakaguchi, 2014).

This poor outcome in cancer may be due to variable mechanisms. Regulatory T cells inhibit many adaptive and innate immune cells, including CD4+ T cells, CD8+ T cells, dendritic cells, macrophages, and B cells. It has been shown that Treg cells also inhibit NK cells in a TGF-b dependent manner (Ralainirina et al., 2007). Also, Treg cells can suppress immune responses of effector T cells as well as other immune cells through direct cell-cell contact-dependent mechanisms and release of various soluble factors (Schmidt et al., 2012).

Another explanation, Tregs can induce immune tolerance and lead to tumor progression by the following mechanisms: secretion of immunosuppressive molecules such as transforming growth factor beta (TGFβ), IL-10 and CCL22; directly cytolysis of NK cells and CD8+ cells; metabolic disruption; and promoting angiogenesis (Gupta et al., 2007; Ohara et al., 2009; Facciabene et al., 2012; Devaud et al., 2014). On the other hand, Tregs can inhibit tumor-promoting inflammation induced by bacteria infection, thereby contributing to an improved outcome (Ladoire et al., 2011).

Tumor infiltrating lymphocytes (TILs) may be classified into lymphocytes infiltrating the tumor cell islets (thus being in direct contact with tumor cells) called intra-epithelial (intra-tumoral) and TILs lymphocytes infiltrating the tumor stroma (peritumoral infiltrating lymphocytes) (Hornychova et al., 2008).

Sakaguchi et al., 2008 demonstrated that tumor infiltrating lymphocytes (TILs) are composed of different kinds of lymphocytes, including CD4+ and CD8+ T cells, Tregs, B cells, NK cells and NKT cells. Treg cells expressing the transcription factor FOXP3 are naturally present in the immune system and their dysfunction due to FOXP3 gene mutation causes fatal autoimmune disease (Sakaguchi et al., 2008).

Most FOXP3+ Treg cells are CD4+ T cells that express CD25 can suppress the activation, proliferation and effector functions of a wide range of immune cells displaying a central role in the prevention of immune diseases (Sakaguchi et al., 2008).

In the current study, tumoral FOXP3 H. Score was significantly associated with an increase in peritumoral positively stained FOXP3+ lymphocytes H- score (p=0.001) and this agrees with a study done by (Allan & Hafler 2006) who reported that a high infiltration of FOXP3+ lymphocytes was accompanied by FOXP3+ tumor expression in cancer breast. FOXP3 expressed in the malignant cells succeeded to recruit lymphocytes infiltration into tumor microenvironment (Allan & Hafler, 2006; Takenaka et al., 2013). Regarding the overall survival, in the current study, the presence of bilharziasis, advanced pathological T stage and presence of lymph node invasion showed poor impact on patient's outcome. Furthermore, by multivariate COX- regression analysis, the pathological T stage was the most independent prognostic factor affecting patient's overall survival agreeing with (Moch et al., 2016).

On contrary with several studies found that Trop2 expression had been associated with poor survival in various cancers including; CRC (Xu and Gu et al., 2009; Fang et al., 2009; Sukunthankar et al., 2010), breast cancer (Huang et al., 2005), gastric cancer (Muhlmann et al., 2009; Zhao et al., 2016; Zhao et al., 2017) and cancer cervix (Liu et al., 2013), our study failed to reveal a significant association between the Trop2 immunoreactivity and patient's survival. On the other hand, studies as (Pak et al., 2012) found that Trop2 overexpression was associated with better survival in non-small cell lung cancer (NSCLC) in patients with adenocarcinoma and may be a better prognostic marker in advanced stage adenocarcinoma.

Similarly, our study failed to reveal a significant association between the Cyclin D1 immunoreactivity and patient's overall survival. This was similar to (Sgambato et al., 2002), meanwhile (Khabaz et al., 2016) reported that

decreased expression of Cyclin D1 was associated with poor prognosis.

Furthermore, our study failed to reveal a significant association between the tumoral FOXP3 immunoreactivity and patient's survival. This was contrary to (Hinz et al., 2007; Zhang et al., 2016; Merlo et al., 2009; Peng et al., 2019) who found that increased expression of FOXP3 was associated unfavourable prognosis in pancreas, bladder and breast carcinoma respectively which is in contrast to (Ladoire et al., 2011; Ma et al, 2014) who demonstrated a positive correlation between tumoral FOXP3 and survival in patients with HER2+ tumours who have received neoadjuvant therapy and in gastric carcinoma respectively. Findings in the current study might be explained by the small sample size of the studied cases.

An inverse relationship between Cyclin D1 H-score and both Trop2 and FOXP3 H-score in primary bladder carcinoma cases, in the current study, was demonstrated, as Cyclin D1 served as a good prognostic marker, while, Trop2 and FOXP3was an unfavorable element of bladder cancer patients. However, no previous studies assessed this relationship in the malignant tumor, yet this inverse relationship could be explained as proposed by a study done by (Guerra et al., 2013) , that the phosphorylated forms of Cyclin D1are faster-migrating forms having a shorter half-life and that Trop2 decreases Cyclin D1 expression overall.Trop2 forms an oncogenic chimeric Cyclin D1- Trop2 protein, implicated in cell transformation in urinary bladder carcinoma which eventually consumes the Cyclin D1 causing the inverse relationship between both Trop2 and Cyclin D, is a proposed explanation for the inverse relationship in the current study.

CONCLUSION

Both Trop2 and FOXP3 imply poor prognostic impact and predicts tumor aggressiveness in bladder carcinoma in Egyptian patients while Cyclin D1 implies a favorable one. The inverse relation between Cyclin D1 and Trop2 proposes the consumption of Cyclin D1 by Trop2 as a ligand in the urinary bladder carcinogenesis in bladder carcinoma with high grade, advanced stage, high mitotic count and muscle invasion. Trop2, FOXP3 and Cyclin D1 are suggested to be promising candidate biomarkers that might serve as prognostic factors for the prediction of tumor behavior in bladder carcinoma in Egyptian patients. Further studies conducted on a larger scale to convey the role of Trop2 and FOXP3 antagonists as potential targeted therapeutic tools in UBC, is recommended.

List of abbreviations

BC: Bladder cancer
Tregs : regulatory T lymphocytes
AJCC : American Joint Committee on Cancer
AJCC-UICC: American Joint Committee on Cancer-Union International Center Cancer staging system
NMIBC: Non-muscle- invasive bladder cancer
MIBC: Muscle invasive bladder cancer
UC: Urothelial carcinoma
IHC: immunohistochemical
IRS : immunoreactive score
PNI: perineural invasion
TILs :Tumor infiltrating lymphocytes

DECLARATIONS

The ethics committee in the faculty of medicine Menoufia University approved the purpose of this retrospective study and the use of archival paraffin blocks to evaluate the expression of the studied primary antibodies.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from [third party name] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [third party name].

CONFLICTS OF INTEREST

All authors have approved this article and declare no conflicts of interest.

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