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 3:1 male to female ratio. It comprises 88.3% of the total urinary system tumors. To reduce bladder cancer morbidity and mortality there is an urgent need to identify novel tumor marker which are specific enough for prognosis and can serve as effective anticancer targets. Therefore the purpose of this study is to evaluate the role of TROP2, CYCLIN D1, FOXP3 and their relationship with the established clinico-pathological parameters and overall survival of bladder cancer in Egyptian patients

Methods

Using the standard immunohistochemical technique, TROP2, CYCLIN D1 and FOXP3 expression in 80 primary bladder carcinomas and 20 specimens as non neoplastic group were assessed. The bladder carcinoma cases included 50 cases with muscle invasive bladder cancer and 30 cases with non-muscle invasive bladder cancer

Results

Overexpression of both TROP2 and FOXP3 implied poor prognostic impact as significantly associated with muscle invasive bladder cancer, high grade, advanced stage, lymph node involvement and high mitotic count. Cyclin D1 displayed an inverse relation with both TROP2 and FOXP3 reflecting a favorable prognostic impact. Tumoral FOXP3 expression is directly correlated with peritumoral FOXP3+ lymphocytes expression. 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 consumption of Cyclin D1 by TROP2 as a ligand in the urinary bladder carcinogenesis. Strong diffuse overexpression of both TROP2, and FOXP3 could be promising potential biomarkers for identifying patients with poor prognostic factors in bladder cancer serving as potential targets for cancer therapy.

Background

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 **(1)**. The expected new cases are about 10.709 by 2020, 12.762 by 2025 and 28.337 by 2050 **(2,3)**.

To reduce bladder cancer morbidity and mortality there is an urgent need to identify novel tumor marker which are specific enough for prognosis and can serve as effective anticancer targets **(4)**.

TROP-2 is a transmembrane glycoprotein encoded by the *Tacstd2* gene **(5)**, which has been actively studied as a prognostic marker and an attractive immunotherapeutic target in human cancer treatment **(6)**.

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 (7). Trop2 forms an oncogenic fusion protein with cyclin D1 (8). This chimera is expressed by human tumors differentially (9). FOXP3 is a forkhead box transcription factor containing a DNA-binding domain (10), it is known as the most specific marker of the regulatory T lymphocytes (Tregs) (11). FOXP3 plays a crucial role in the development and function of Tregs, it is constitutively expressed in the nucleus of human Tregs (12).

The aim of this study is to investigate 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 included 50 cases with MIBC and 30 cases of NMIBC. 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

The hematoxylin and eosin (H&E) stained sections were evaluated for the followings; Histological type according to WHO classification, 2016. Tumor grading was done according to WHO/ISUP grading criteria (13)

Mitotic and apoptotic count were counted semi quantitatively in ten randomly selected high power fields (14) 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 (NMIBC) (stage pTa and pT1) or muscle invasive urinary bladder carcinoma (MIBC) (stage pT2, pT3 and pT4) (15).

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 polyclonal 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 positive control for TROP-2 (16), from normal human tonsil for Cyclin D1 (17) and from 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 **(16)**. Nuclear staining in any number of cells for Cyclin D1 were required to assign the positivity **(17)** FOXP3 was assessed in malignant epithelial tissues and in 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 **(18)**.

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 **(19)**.

Results

-Clinicopathologic characteristics:

Clinicopathologic characteristics of primary bladder carcinoma cases (80 cases) are summarized in **[table 1]**, as for the clinical data of the non neoplastic group (no. =20) their age ranged from 30 to 77 years with mean $\pm$ 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.

-Immunohistochemical (IHC) profile of TROP2, Cyclin D1 and FOXP3 in the studied non neoplastic and UBC groups were summarized in [table 2].

-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.

-Relationship between TROP2, Cyclin D1 and FOXP3 IRS score and clinicopathological factors in primary bladder carcinoma cases [table 3, 4 and 5]

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].

Cyclin D1 high Immunoreactive score (IRS) was correlated with early stage group ($P=0.001$), absence of lymph node involvement ($P=0.001$) and low mitotic count ($P=0.007$). Moreover, all high IRS cyclin D1 cases (20/20) displayed absence of bilharzial infestation ($P=0.001$) [Table 4].

Regarding tumoral FOXP3 immunoreactive score (IRS), low IRS was in favor of early stage group ($P=0.003$) and absence of lymph node involvement ($P=0.025$). In contrast, high FOXP3 IRS was significantly in favor of presence of LVI, since 9 cases out of 17 cases of positive LVI displayed high IRS ($P=0.001$). Also, high FOXP3 IRS was statistically associated with high mitotic count ($P=0.008$) [Table 5].

-Correlation between Cyclin D1 H-score and both TROP2 and tumoral FOXP3 H-scores in malignant cases

There was a significant inverse relationship between Cyclin D1 H-score and both TROP2 and FOXP3 H-scores ($P=0.001$ and 0.005 respectively)

-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 ($P=0.044$) (figure 65) on patient's outcome.

None of TROP2, Cyclin D1, tumoral and peritumoral FOXP3 expressions showed significant impact on overall survival

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 (20, 21, 22, 23). Transcription factors known to be involved in cancer cell progression, such as WT1 regulate Trop-2 transcription (24), in addition, overexpression of TROP2 may be due to Trop-2's intrinsic regulatory effects on cancer cell growth, invasion, and proliferation (25). So, overexpression of Trop-2 naturally leads to tumor progression as a key driver of cancer growth (6). On the other hand, Trop2 was found to be over expressed across normal tissues in animal model, including bladder, uterus, kidney, lung, and skin (23). 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 (26, 27). Trop2 shares a conserved cysteine-rich region in its extracellular domain that is required for EpCAM-mediated adhesions (27).

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 (20) who demonstrated that high expression of TROP2 and high score were significantly associated with tumor high grade, advanced stage, and recurrence in UBC **who also** found that TROP2 overexpression was associated with tumor stage, lymph node involvement, tumor size and distant metastases in gastric carcinoma.

Studies such as (28,29,4) found that overexpression of TROP2 enhanced cell proliferation, migration, and invasion in the lung cancer cells, gall bladder cancer and oral squamous cell carcinoma (OSCC) respectively, while downregulation of TROP2 triggered apoptosis and impaired proliferation. This was explained by (30,29) who found that TROP2 lead to activation and regulation of ERK pathway in cervical cancer cells and regulation of PI3K/AKT pathway inducing EMT in gall bladder cancer respectively. Moreover, **studies done (31,32)** found that loss of TROP2 lead to autocrine activation of the EGFR family member ErbB3 through neu-

regulin-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 concordance with (33) who reported cyclin D1 immunoreactivity similarly frequent in bladder tumors (51.6%) and normal tissue of bladder (50%). A study done in 2007 (34) agreeing, reported uniformly intense expression of cyclin D1 in the non-neoplastic group. However other studies (35) reported higher cyclin D1 protein expression in UBC *and in endometrial carcinoma (36) compared to* the adjacent normal tissue.

While other studies (37, 38, 39) reported complete absence of cyclin D1 in normal urothelium and in other tissues as colonic and gastric mucosa (40, 41, 42) while only expressed in the carcinoma group.

Cyclin D1 high expression was reported in early stage group ($P=0.031$), absent PNI ($P=0.037$) and absence of lymph node involvement ($P=0.001$), in agreement with (43,44,17) who stated low level of Cyclin D1 in advanced stage, poorly differentiated tumors, vascular invasion, as well as lymph node involvement. While Lee *et al., 2010* found that cyclin D1 was significantly higher with advanced stage and MIBC (45).

In the current study , all high IRS cyclin D1 cases (20/20) displayed absence of bilharzial infestation ($P=0.001$) in agreement with which was explained by (46) . The favorable prognostic impact implied 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 (47) *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 (48). 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 (17)

The inverse correlation between Cyclin D1 expression and poor prognostic parameters was not only reported in urothelial carcinoma; but also among other tumors, as in gastric carcinoma (49), in laryngeal squamous cell carcinoma (50) and in invasive breast carcinoma (51).

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

In this current study, FOXP3 was expressed in 85% of the normal urothelium and in 75% of the tumor cells in malignant group ($P>0.05$). This is in contrast to (18) and (52) who found that FOXP3 was highly expressed in cancer cells of UBC while (53) found that FOXP3 was expressed in normal breast and down-regulated in adjacent mammary cancer. Predominance of missense mutations in breast and prostate cancer patients via transcriptionally repressing ErbB2 and Skp2 genes in breast cancer and c-Myc gene in prostate cancer suggested the role of FOXP3 as a tumor suppressor gene (53, and 54).

Low tumoral FOXP3 immunoreactive score (IRS) is in favor of early stage group ($P=0.003$), absence of lymph node involvement ($P=0.025$), absence of LVI ($P=0.001$) and low mitotic count ($P=0.008$). Association of tumoral FOXP3 with poor prognostic factors is in agreement with (55) who demonstrated that expression of

tumoral FOXP3 was associated with lymphatic metastasis, advanced stage and high proliferative index (Ki-67 $\geq 14\%$) 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 which have been observed in cell lines derived from breast, prostate and ovarian cancers that overexpress FOXP3 (56).

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- β dependent manner (57). 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 (58).

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 (TGF β), IL-10 and CCL22; directly cytotoxicity of NK cells and CD8+ cells; metabolic disruption; and promoting angiogenesis (59; 60; 61; 62). On the other hand, Tregs can inhibit tumor-promoting inflammation induced by bacteria infection, thereby contributing to an improved outcome (63).

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) (64).

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 (65).

Most of 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 (65,66).

In this current study, tumoral FOXP3 H. Score was significantly associated with increase in peritumoral positively stained FOXP3+ lymphocytes H. score ($p=0.001$) and this agrees with (67) 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 .Most of FOXP3+ Treg cells are CD4+ T cells that express CD25 suppressing effector functions of a wide range of immune cells.

Regarding overall survival, in current study, 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, pathological T stage was the most independent prognostic factor affecting patient's overall survival agreeing with (13).

On contrary with several studies which found that TROP2 expression had been associated with poor survival in various cancers including; CRC (**68, 69** and **70**), breast cancer (**8**), gastric cancer (**71; 72; 73**) and cancer cervix (**30**), our study failed to revealed significant association between the TROP2 immunoreactivity and patient's survival. On the other hand, studies as (**74**) 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 significant association between the Cyclin D1 immunoreactivity and patient's overall survival. This was similar to (**75**), while (**33**) reported that decreased expression of Cyclin D1 was associated with poor prognosis.

Furthermore, our study failed to reveal significant association between the tumoral FOXP3 immunoreactivity and patient's survival. This was contrary to (**76, 52; 77, 55**) who found that increased expression of FOXP3 was associated unfavourable prognosis in pancreas, bladder and breast carcinoma respectively which is in contrast to (**63,78**) 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. These findings might be explained by few number of studied cases.

The present study showed a significant inverse relationship between Cyclin D1 H-score and both TROP2 and FOXP3 H-score in primary bladder carcinoma cases, as Cyclin D1 served as a good prognostic marker, while, TROP2 and FOXP3 was an unfavorable element of bladder cancer patients. However, no previous studies assessed this relationship in malignant tumor, yet this inverse relationship could be explained as proposed by (**6**) that the phosphorylated forms of cyclin D1 are faster-migrating forms having a shorter half-life and that Trop-2 decreases cyclin D1 expression overall .In the current study, 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 relation between both Trop2 and cylin D.

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 a promising candidate biomarkers that might serve as prognostic factors for 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.

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

Ethics approval and consent to participate:

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.

Consent for publication:

Not applicable

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].

Competing interests:

The authors declare that they have no competing interests

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Authors' contributions:

Dalia Rifaat Al-Sharaky - participated in histopathological assesement of the stained slides, interpretation of the results, analysis and a contributor in writing the manuscript.

Moshira Mohammed Abdelwahed - participated in interpretation of the results, analysis and writing the discussion.

Hend Ahmad Abdou Kassem- participated in histopathological assesement of the stained slides, interpretation of the results, analysis and writing the manuscript.

Abdelnaby Saied Abdelnaby - participated in histopathological assesement of the stained slides

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Tables

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	Male	No	%
	Female	66	82.5
		14	17.5
Tumor Size (50 cases)	Mean±SD	4.7±1.2	
	Range	4.7	
	Median	3-9	
WHO Histologic Type (2016)	Infiltrating UC	NO	%
	Noninvasive papillary UC	76	95
		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	Dysmoplastic	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

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	17	85	78	97.5	FE=5.6	0.056
	Negative	3	15	2	2.5		
		No=17		No=78			
Percentage	Mean±SD	64.1±15.9		72.0±19.2		FE=1.7	0.081
	Range	30– 90		20 – 100			
	Median	70		77.5			
TROP2 Subcellular localization	Membranous Membranocytoplasmic	0 17	0 100	52 26	66.7 33.3	FE=7.8	0.005*
TROP2 predominant intensity	Mild	0	0	10	12.8 34.6	FE=4.1	0.128
	Moderate	4	23.5	27	52.6		
	Strong	13	76.5	41			
TROP2 pattern of staining	Patchy	5 12	29.4 70.6	19	24.4	FE=0.189	0.759
	Diffuse			59	75.6		
TROP2 IRS	Low	6	35.3 64.7	31	39.7 60.3	χ ² =0.116	0.733
	High	11		47			
TROP2 H Score	Mean±SD	174.1±56.3		182.5±73.9		FE=0.696	0.487
	Median	50 – 240		20 – 300			
	Range	180		190			

Cyclin D1 expression	Positive	17	85	61	76.2	FE= 0.714	0.551
	Negative	3	15	19	23.8		
		No=17		No=61			
Percentage	Mean± SD	39.4±21.5		48.4±21.6		U= 1.5	0.123
	Range	10 – 80		10 – 90			
	Median	40		50			
Predominant intensity	Mild	NO	%	NO	%	FE= 1.7	0.425
	Moderate	2	11.8	10	16.4		
	Strong	10	58.8	25	41		
		5	29.4	26	42.6		
Cyclin D1 IRS	Low	15	88.2	41	67.2	FE=2.9	0.129
	High		11.8	20	32.8		
Distribution	Patchy	11	64.7	43	70.5	x²=0.209	0.648
	Diffuse	6	35.3	18	29.5		
Cyclin D1 H.Score	Mean± SD	88.5±58.1		111.1±59.4		U=1.4	0.123
	Range	20 – 220		10 – 240			
	Median	80		120			

FOXP3 Expression	negative	NO	%	NO	%	FE= 0.903	0.342
	positive	20	25	3	15		
		60	75	17	85		
		NO=60		NO=17			
FOXP3 Percent	Mean± SD	40.0±21.7		42.9±18.2		U= 0.720	0.471
	Range	10– 80		10– 60			
	Median	30		50			
FOXP3 Distribution	Patchy	54	90	11	64.7	FE= 6.44	0.011*
	Diffuse	6	10		35.3		
FOXP3 Intensity	Mild to moderate	42	70	16	94.1	FE= 5.6	0.060
	strong	18	30	1	5.9		
FOXP3 Subcellular localization	cytoplasmic	60	100	9	52.9	FE= 31.5	0.001*
	apical cytoplasmic	0	0	8	47.1		
FOXP3 IRS	Low	46	6.7	15	88.2	FE= 1.077	0.299
	High	14	23.3	2	11.8		
FOXP3 H score	Mean± SD	81.3±53.7		69.4±30.7		U= 0.351	0.726
	Range	10 – 200		20 – 120			
	Median	70		60			

Table 3: Relationship between TROP2 immunoreactive score (IRS) and studied clinico-pathological parameters in malignant cases

		TROP2 IRS				test of significant	P value
		Low		High			
		(no=31)		(no=47)			
		NO	%	NO	%		
Gender	Male (no=64)	24	37.5	40	62.5	χ 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
	Non invasive papillary UC (no=3)	1	33.3	2	66.7		
Grade	Low (no=17)	11	64.7	6	35.3	x ² =5.6	0.017*
	High (no=61)	20	32.8	41	67.2		
stage group	Early (no=28)	24	85.7	4	14.3	x ² = 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	x ² = 0.335	0.563
	present (no=23)	8	34.8	15	65.2		
Necrosis	absent (no=50)	19	38	31	62	0.177 (x ²)	0.674
	present (no=28)	12	42.9	16	57.1		
LVI	absent (no=58)	30	51.7	28	48.3	x ² =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*
		0	0	10	100		

	present (no=10)						
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		
		4	30.8	18	62.1		
	both (no=22)						
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		High			
		(no=41)		(no=20)			
Gender	Male (no=50)	NO	%	NO	%	FE=0.977	0.323
	Female (no=11)	35	67.3	15	32.7		
		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	FE=15.7	0.001*
	Present (no=20)	17	85	3	15		
Bilharasis	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		High			
		(no=46)		(no=14)			
Gender	Male (no=51)	NO	%	NO	%	FE= 0.007	0.932
	Female (no=9)	39	76.5	12	23.5		
		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
	Non invasive 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	(x²)= 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		