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Expression Of TIM-3 And CEACAM-1 In Bladder Urothelial Carcinoma (Immunohistochmical And Histopathological Study)

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Abstract:

Background and objectives: Urothelial carcinoma is now considered to be the most important tumor of the urinary bladder in Egypt especially after decreased prevalence of schistosomiasis. Due to few available therapeutic approaches and partial success in improving patients' survival, novel immunotherapeutic agents such as immune checkpoint inhibitors become a promising hope especially after PD-1 and CTLA-4 being clinically relevant. TIM-3 which is another immune checkpoints molecule is recently appeared to be associated with immune suppression in many tumors and has been reported to be aberrantly expressed in several human malignancies including urothelial carcinoma. One of the TIM-3 discovered ligands is CEACAM-1. The latter has been linked to malignancy progression and metastatic spread and has been expressed in many tumor types. The aim of our work is to evaluate the expression of TIM-3 and CEACAM-1 in bladder urothelial carcinoma and analyse their correlations with clinical and pathological related factors. **Methods:** Different TIM-3 and CEACACM-1 expressions were evaluated in immunohistochemically stained paraffin embedded sections from 60 Egyptian patients with bladder urothelial carcinoma. Expression was correlated with the available clinical and pathological data. **Results:** Both TIM-3 and CEACAM-1 were expressed in cancer cells, tumor infiltrating lymphocytes and endothelial cells. TIM-3 expression by cancer cells showed significant correlation with tumor necrosis and TIM-3 expression by TILs. TIM-3 expression by TILs significantly related to TIM-3 expression by endothelial cells. Endothelial CEACAM-1 positivity significantly correlated to tumor grade, associated inflammation, tumor necrosis, muscle invasion and tumor

differentiation. CEACAM-1 expression by TILs was significantly related to its expression in tumor cells and absent vascular emboli. Regarding correlations between the both studied markers, expression of TIM-3 by cancer cells was significantly correlated to CEACAM-1 expression by both TILs and endothelial cells. In addition, TIM-3 and CEACAM-1 expressions by TILs were significantly positively correlated. **Conclusion:** The expressions of TIM-3 and CEACAM-1 in urothelial cancer cells and TILs suggest a potential role for these molecules in cancer development and progression and introduce them as a novel targets in bladder cancer therapy. In addition, endothelial CEACAM-1 expression could be a marker of tumor progression and invasiveness.

Keywords: TIM-3, CEACAM-1, urothelial carcinoma, immunotherapy**.**

1. Introduction:

Urothelial carcinoma (UC) of the urinary tract is one of the top ten most common types of malignancy worldwide and the urinary bladder is the most common pathologic site of occurrence **(1)**. In Egypt, bladder cancer is a big health burden where it is one of the commonest cancers **(2).** UC is well recognized as an immunogenic and immunoresponsive tumor and the intravesicle bacillus calmette-Guerin (BCG) therapeutic strategy is a well-defined proof of that **(3).**

The expression of immune checkpoint proteins is considered one of the tumor mechanisms to evade immune surveillance and promote immune tolerance. In recent years, several different monoclonal antibodies directing these checkpoints have been approved by the Food and Drug Administration (FDA) for UC **(4).**

A number of novel immune checkpoints targets currently are under investigation like T-cell immunoglobulin mucin-3 (TIM-3) that may soon emerge as important treatment options for patients with UC. TIM-3 is considered an activation-induced inhibitory molecule that is found to be expressed on activated human T cells, NK cells, and monocytes. It has a role in immune tolerance and revealed to induce T-cell exhaustion in chronic viral infection and cancers **(5)**.

In bladder UC, TIM-3 has appeared to have an important role in its aggressive growth by antagonizing anti-tumor immunity and inducing tumor progression at several levels. These findings have suggested that TIM-3 expression could be a poor prognostic variable for patient survival and introducing it as a potential biomarker intended for UC therapies development **(6).**

Carcinoembryonic antigen cell adhesion molecule 1 (CEACAM-1) has been emerged as a heterophilic TIM-3 ligand required to mediate T-cell inhibition and this interaction has been found to be crucial in autoimmunity and anti-tumor immunity control **(7).** In addition, a decade ago, CEACAM-1 has been reported as a novel

urinary marker for bladder cancer with urinary levels increasing with advancing stage and grade **(8)**.

The co-expression of TIM-3 and CEACAM-1 in colorectal cancer has been studied and the results suggest that both can mediate T cell exhaustion and can be potential biomarkers for colorectal cancer prediction **(9).** Furthermore, all anti-TIM-3 antibodies that have proved functional efficacy in mouse cancer models have been found to interfere with CEACAM-1 binding. Thus, the TIM-3–CEACAM-1 axis has the potential to be an important target for immunotherapy for cancer **(10)**.

2. Materials and Methods:

The material of this study consisted of 60 urothelial carcinoma specimens; 37 obtained by trans-urethral resection and 23 by radical cystectomies, collected retrospectively and prospectively from the pathology lab of Beni-Suef university hospital in the form of formalin fixed paraffin embedded tissue blocks. Demographic and pathological data were obtained from patients' reports.

Inclusion criteria were primary bladder urothelial carcinoma treated either with radical cystectomy or transuretheral resection, adequate viable tumor tissue, available clinical data. Exclusion criteria included secondary urothelial carcinoma or bladder squamous cell carcinoma.

Histopathologic study

The H&E sections were re-evaluated for the tumor type and its grade according to the WHO 2016 histological classification of tumors of the urinary tract **(11)**. The depth of tumor invasion in ***56 cases only (as 4 cases were without submitted muscle)***, associated bilharziasis, presence of tumor necrosis, degree of associated inflammatory response and perineural and vascular invasion were also re-evaluated. In the 23 radical cystectomy specimens, tumors were staged according to the 8th edition of Tumor Node Metastasis TNM system of the American Joint Committee on Cancer (AJCC) **(12)** and the lymph node status were mentioned as negative or positive.

Immunohistochemical (IHC) study:

Immunohistochemical studies were performed on five-micron sections prepared from formalin-fixed and paraffin embedded tissue using standard autostaining protocols on a Ventana Benchmark XT autostainer (Ventana Medical Systems, Inc. Tucson, AZ). Deparaffinization and antigen retrieval (i-view detection system; Ventana) were carried out as an automated program of the Ventana autostainer. The primary antibodies were polyclonal TIM3 antibody (Catalogue No.:abx225210, Abbexa, Cambridge, UK) and polyclonal CEACAM1 antibody (Catalogue No.:abx104701, Abbexa, Cambridge, UK).

In each staining session for each TIM3 antibody and CEACAM-1 antibody, a section from the tonsil previously known to be positive for both of each was used as a positive control. As a negative control, a tissue section was processed in the above mentioned sequence, but the primary antibody was not added and instead PBS was used in this step.

Slides examination and imaging:

All slides were examined by both light microscopy and by the virtual microscopy (Leica, APERIO LV1) in the pathology lab of Beni-Suef university hospital. All included photos were imaged by the virtual microscope.

Interpretation of TIM3 immunostaining

The tumor tissue sections were examined for the expression of TIM-3 in tumor cells, TILs and endothelial cells. For tumor cells evaluation, five visual fields were randomly selected in each section, and 200 tumor cells were counted in each visual field at x200 magnification. Cells were regarded as positive if the cell membrane and/or cytoplasm stained brown. The percentage of positive cells and the intensity of staining were evaluated in all sections, and the final score for each section was derived from the multiplication of the two.

The scoring system for the percentage of positive cells was as follows: 1 point: ≤33%; 2 points: >33 to ≤66%; 3 points: >66%. The scoring system for staining intensity was as follows: 1 point: absent/weak staining; 2 points: moderate staining; 3 points: strong staining. Sections with a final overall score of ≤3 were classified as the TIM‑3 low expression group; other sections were classified as the TIM‑3 high expression group **(13)**.

TILs were examined in all sections for TIM-3 expression, and were graded as low expression if <20% of cell were positive, high expression if ≥20% and negative if <1% **(14)**. TIM-3 expression by endothelial cells was considered as positive or negative.

Interpretation of CEACAM1 immunostaining

The tumour tissue sections were examined for the expression of CEACAM1 in tumor cells, TILs and endothelial cells. For tumor cells evaluation; cells were regarded as positive if the cell membrane and/or cytoplasm stained brown. A proportion and an intensity scores were assigned to each specimen.

The proportion score represented the estimated proportion of positive tumor cells (0: none; 1: <10%; 2: 10-33%; 3: >33-66% and 4: >66%). The intensity score represented the average intensity of the positive tumor cells (0: none; 1: weak; 2: intermediate; 3: strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 7. All specimens were divided into 3 groups for further statistical analyses (negative/ weak expression: 0–2 points;

moderate expression: 3–4 points; strong expression: 5-7 points) **(15)**.

TILs and endothelial cells were examined in all sections for CEACAM-1 expression, and were graded as done for TIM-3.

Statistical analysis

Data were statistically described in terms of mean ± standard deviation range, frequencies and percentages. Chi-square test was used for comparing categorical data and testing any significant correlation between TIM3 and CEACAM1 expression and other clinicopathological variables included in the study. P value was set significant if it was less than 0.05. All analyses were done using SPSS (Statistical Package for Social Sciences software, version 18, Chicago, IL, USA) release 15 for Microsoft Windows (2006).

3. Results:

The present study revealed significant relations between TIM-3 expression by cancer cells and tumor necrosis (P=0.043) and TIM-3 expression by TILs (P<0.001). In addition, TIM-3 expression by TILs significantly related to TIM-3 expression by endothelial cells (P=0.009). Moreover, endothelial CEACAM-1 positivity significantly correlated to tumor grade (P<0.001), associated inflammation (P=0.030), tumor necrosis (P=0.024), muscle invasion (P=0.001) and tumor differentiation (P<0.001) as being less frequently associated with papillary differentiation. Furthermore, CEACAM-1 expression by TILs was significantly related to its expression in tumor cells (P=0.021) and to absent vascular emboli (P=0.030).

Regarding correlations between the both studied markers, expression of TIM-3 by cancer cells was significantly correlated to CEACAM-1 expression by both TILs (P=0.022) and endothelial cells (P=0.029). In addition, TIM-3 expression by TILs showed significant relation with CEACAM-1 expression by TILs (P<0.001).

Finally, no significant relations found between different TIM-3 and CEACAM-1 expressions and age, sex, tumor size, bilharziasis, perineural invasions, tumor stage and lymph node metastasis.

Parameters		CEACAM-1 expression by tumor cells				TIM-3 expression by tumor cells		
	Total (60) N	Negative/weak (6) N %	Moderate (12) N %	Strong (42) N %	*P*-value	Low (25) N %	High (35) N %	*P*-value
Age								
< 65	25		6 (24%)	15 (60%)	0.287	14 (56%)	11 (44%)	0.757
≥ 65	35	4 (16%)	6 (17.1%)	27 (77.1%)		14 (40%)	21 (60%)	
		2 (5.7%)						
Sex								
Male	46	6 (13%)	10 (21.7%)	30 (65.2%)	0.251	29 (63%)	17 (37%)	0.180
Female	14	0 (0%)	2 (14.3%)	12 (85.7%)		6 (42.9%)	8 (57.1%)	
Tumor size								
< 3 cm	19	1 (5.3%)	1 (5.3%)	17 (89.4%)	0.075	12 (63.2%)	7 (36.8%)	0.606
≥ 3 cm	41	5 (12.2%)	11 (26.8%)	25 (61%)		23 (56.1%)	18 (43.9%)	
Tumor grade								
Low	10	1 (10%)	2 (20%)	7 (70%)	1	6 (60%)	4 (40%)	0.198
High	50	5 (10%)	10 (20%)	35 (70%)		19 (38%)	31 (62%)	
Bilharziasis								
Absent	39	4 (10.3%)	6 (15.4%)	29 (74.4%)	0.473	17 (43.6%)	22 (56.4%)	0.681
Present	21	2 (9.5%)	6 (28.6%)	13 (61.9%)		8 (38.1%)	13 (61.9%)	
Inflammation								
Mild	21	3 (14.3%)	4 (19%)	14 (66.7%)	0.939	10 (47.6%)	11 (52.4%)	
Moderate	23	2 (8.7%)	5 (21.7%)	16 (69.6%)		11 (47.8%)	12 (52.2%)	
Marked	16	1 (6.3%)	3 (18.8%)	12 (75.0%)		4 (25.0%)	12 (75%)	0.287
Tumor necrosis								
Absent	34	3 (8.8%)	6 (17.6%)	25 (73.5%)	0.798	18 (52.9%)	16 (47.1%)	0.043*
Present	26	3 (11.5%)	6 (23.1%)	17 (65.4%)		7 (26.9%)	19 (73.1%)	
Vascular emboli								
Absent	46	5 (10.9%)	9 (19.6%)	32 (69.6%)	0.917	18 (39.1%)	28 (60.9%)	0.470
Present	14	1 (7.1%)	3 (21.4%)	10 (71.4%)		7 (50%)	7 (50%)	
Perineural invasion								
Absent								
Present	46	5 (10.9%)	9 (19.6%)	32 (69.6%)	0.917	20 (43.5%)	26 (56.5%)	0.606
	14	1 (7.1%)	3 (21.4%)	10 (71.4%)		5 (35.7%)	9 (64.3%)	

Table 1: Correlations between TIM-3 and CECAM-1 expression in tumor cells and some clinicopathological findings

Parameters		TIM-3 expression by TILs				CEACAM-1 expression by TILs			
	Total (60) N	Negative (4) N %	Low (18) N %	High (38) N %	*P*-value	Negative (2) N %	Low (16) N %	High (42) N %	*P*-value
Age									
< 65	25	1 (4%)	8 (32%)	16 (64%)	0.772	1 (4%)	8 (32%)	16 (64%)	0.693
≥ 65	35	3 (8.6%)	10 (28.6%)	22 (62.9%)		1 (2.9%)	8 (22.9%)	26 (74.3%)	
Sex									
Male	46	3 (6.5%)	13 (28.3%)	30 (65.2%)	0.854	2 (4.3%)	11 (23.9%)	33 (71.7%)	0.534
Female	14	1 (7.1%)	5 (35.7%)	8 (57.1%)		0 (0%)	5 (35.7%)	9 (64.3%)	
Tumor size									
< 3 cm	19	1(5.3%)	4 (21.1%)	14 (73.7%)	0.523	0 (0%)	4 (21.1%)	15 (78.9%)	0.455
≥ 3 cm	41	3 (7.3%)	14 (34.1%)	24 (58.5%)		2 (4.9%)	12 (29.2%)	27 (65.9%)	
Tumor grade									
Low	10	0 (0%)	4 (40%)	6 (60%)	0.543	0 (0%)	4 (40%)	6 (60%)	0.504
High	50	4 (8%)	14 (28%)	32 (64%)		2 (4%)	12 (24%)	36 (72%)	
Bilharziasis									
Absent	39	2 (5.1%)	12 (30.8%)	25 (64.1%)	0.807	2 (5.1%)	11 (28.2%)	26 (66.7%)	0.508
Present	21	2 (9.5%)	6 (28.6%)	13 (61.9%)		0 (0%)	5 (23.8%)	16 (76.2%)	
Inflammation									
Mild	21	1 (4.8%)	8 (38.1%)	12 (57.1%)		1 (4.8%)	8 (38.1%)	12 (57.1%)	
Moderate	23	3 (13%)	7 (30.4%)	13 (56.5%)	0.306	1 (4.3%)	7 (30.4%)	15 (65.2%)	
Marked	16	0 (0%)	3 (18.8%)	13 (81.3%)		0 (0%)	1 (6.3%)	15 (93.8%)	0.182
Tumor necrosis									
Absent	34	2 (5.9%)	10 (29.4%)	22 (64.7%)	0.949	0 (0%)	9 (26.5%)	25 (73.5%)	0.252
Present	26	2 (7.7%)	8 (30.8%)	16 (61.5%)		2 (7.7%)	7 (26.9%)	17 (65.4%)	
Vascular emboli									
Absent	46	2 (4.3%)	13 (28.3%)	31 (67.4%)	0.316	0 (0%)	12 (26.1%)	34 (73.9%)	0.030*
Present	14	2 (14.3%)	5 (35.7%)	7 (50%)		2 (14.3%)	4 (28.6%)	8 (57.1%)	
Perineural invasion									
Absent	46	2 (4.3%)	12 (26.1%)	32 (69.6%)	0.149	1 (2.2%)	11 (23.9%)	34 (73.9%)	0.409
Present	14	2 (14.2%)	6 (42.9%)	6 (42.9%)		1 (7.1%)	5 (35.7%)	8 (57.1%)	

Table 2: Correlations between TIM-3 and CECAM-1 expression in TILs and some clinicopathological findings

Parameters		TIM-3 expression by endothelial cells			CEACAM-1 expression by endothelial cells		
	Total (60) N	Negative (5) N %	Positive (55) N %	*P*-value	Negative (6) N %	Positive (54) N %	*P*-value
Age							
< 65	25	1 (4%)		0.305		22 (88%)	0.663
≥ 65	35	4 (11.4%)	24 (96%)		3 (12%)	32 (91.4%)	
			31 (88.6%)		3 (8.6%)		
Sex							
Male	46	5 (10.9%)	41 (89.1%)	0.198	4 (8.7%)	42 (91.3%)	0.542
Female	14	0 (0%)	14 (100%)		2 (14.3%)	12 (85.7%)	
Tumor size							
< 3 cm	19	3 (15.8%)	16 (84.2%)	0.155	2 (10.5%)	17 (89.5%)	0.926
≥ 3 cm	41	2 (4.9%)	39 (95.1%)		4 (9.8%)	37 (90.2%)	
Tumor grade							
Low	10	1 (10%)	9 (90%)	0.835	6 (60%)	4 (40%)	0.000*
High	50	4 (8%)	46 (92%)		0 (0%)	50 (100%)	
Bilharziasis							
Absent	39	2 (5.1%)	37 (94.9%)	0.221	5 (12.8%)	34 (87.2%)	0.321
Present	21	3 (14.3%)	18 (85.7%)		1 (4.8%)	20 (95.2%)	
Inflammation							
Mild	21	1 (4.8%)	20 (95.2%)		5 (23.8%)	16 (76.2%)	
Moderate	23	2 (8.7%)	21 (91.3%)	0.698	1 (4.3%)	22 (95.7%)	
Marked	16	2 (12.5%)	14 (87.5%)		0 (0%)	16 (100%)	0.030*
Tumor necrosis							
Absent	34	4 (11.8%)	30 (88.2%)	0.271	6 (17.6%)	28 (82.4%)	0.024*
Present	26	1 (3.8%)	25 (96.2%)		0 (0%)	26 (100%)	
Vascular emboli							
Absent	46	5 (10.9%)	41 (89.1%)	0.198	6 (13.0%)	40 (87.0%)	0.154
Present	14	0 (0%)	14 (100%)		0 (0%)	14 (100%)	
Perineural invasion							
Absent	46	4 (8.7%)	42 (91.3%)	0.854		40 (87.0%)	
Present	14	1 (7.1%)	13 (92.9%)		6 (13.0%)	14 (100%)	0.154
					0 (0%)		

Table 3: Correlations between TIM-3 and CECAM-1 expression in endothelial cells and some clinicopathological findings

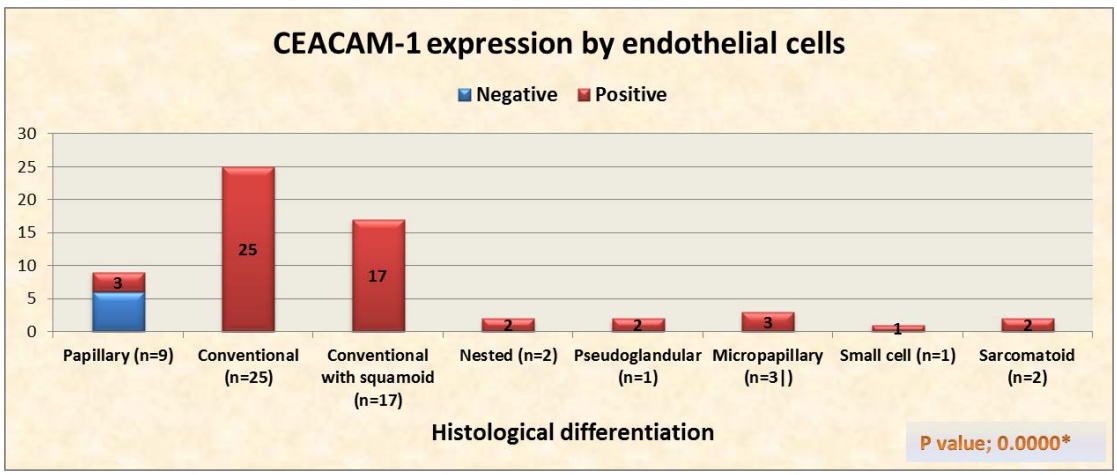
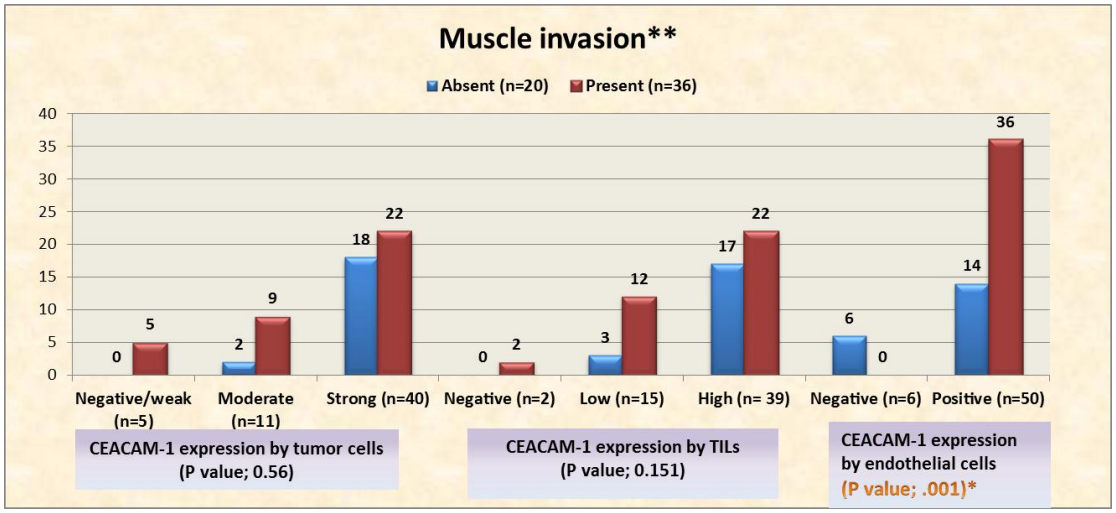


Chart 1: Correlations between histological subtypes and CEACAM-1 expression by endothelial cells



****56 cases as 4 cases were excluded from correlations as they did not have submitted muscularis propria.**

Chart 2: Correlations between muscle invasion and CEACAM-1 expressions

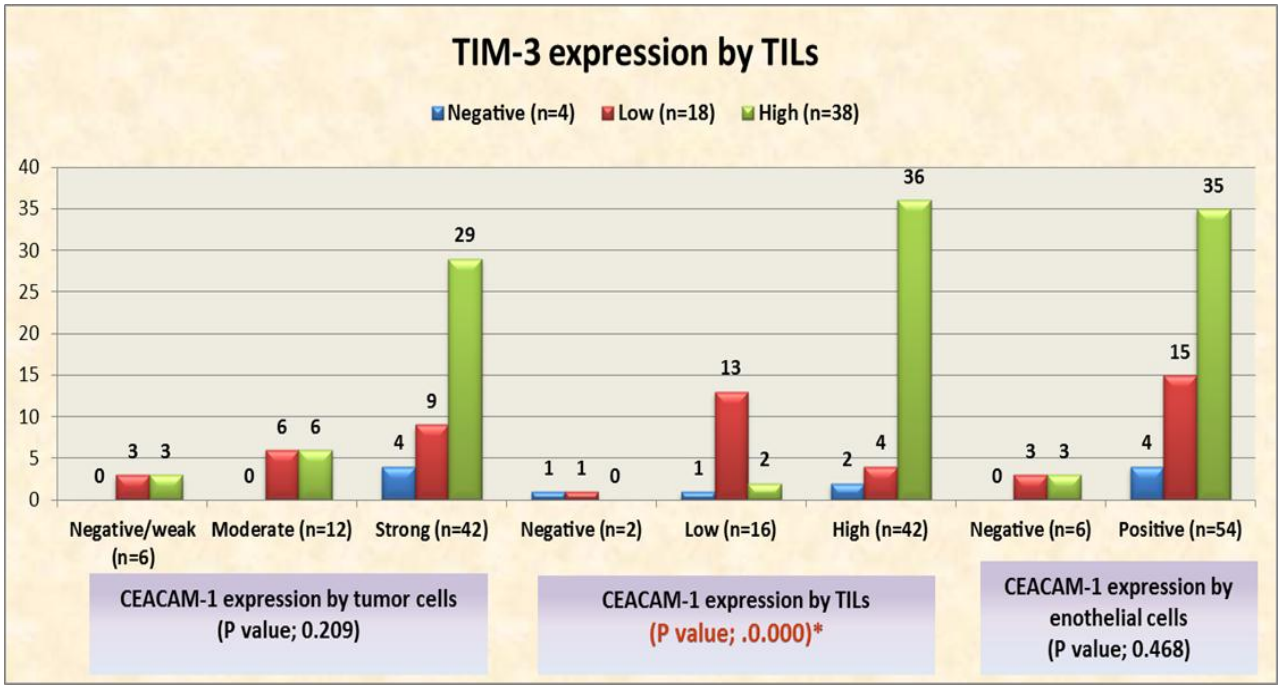


Chart 3: Correlations between TIM-3 expression by TILs and different expressions of CEACAM-1

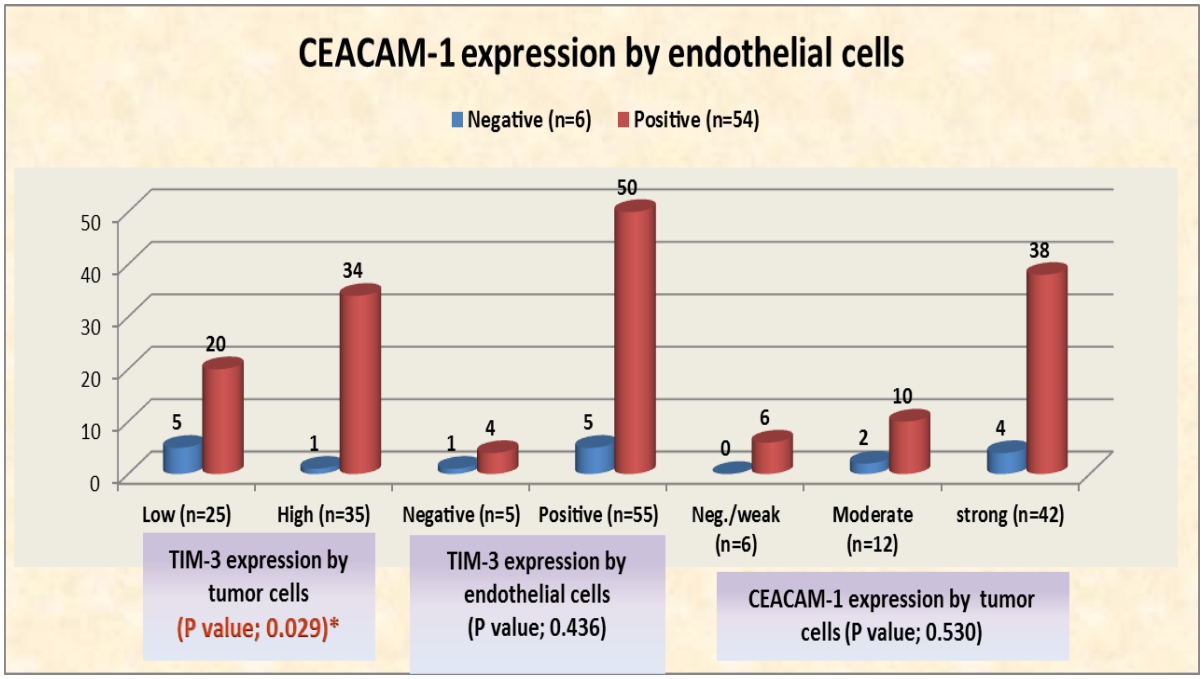


Chart 4: Correlations between CEACAM-1 expression by endothelial cells and other different expressions of TIM-3 and CEACAM-1

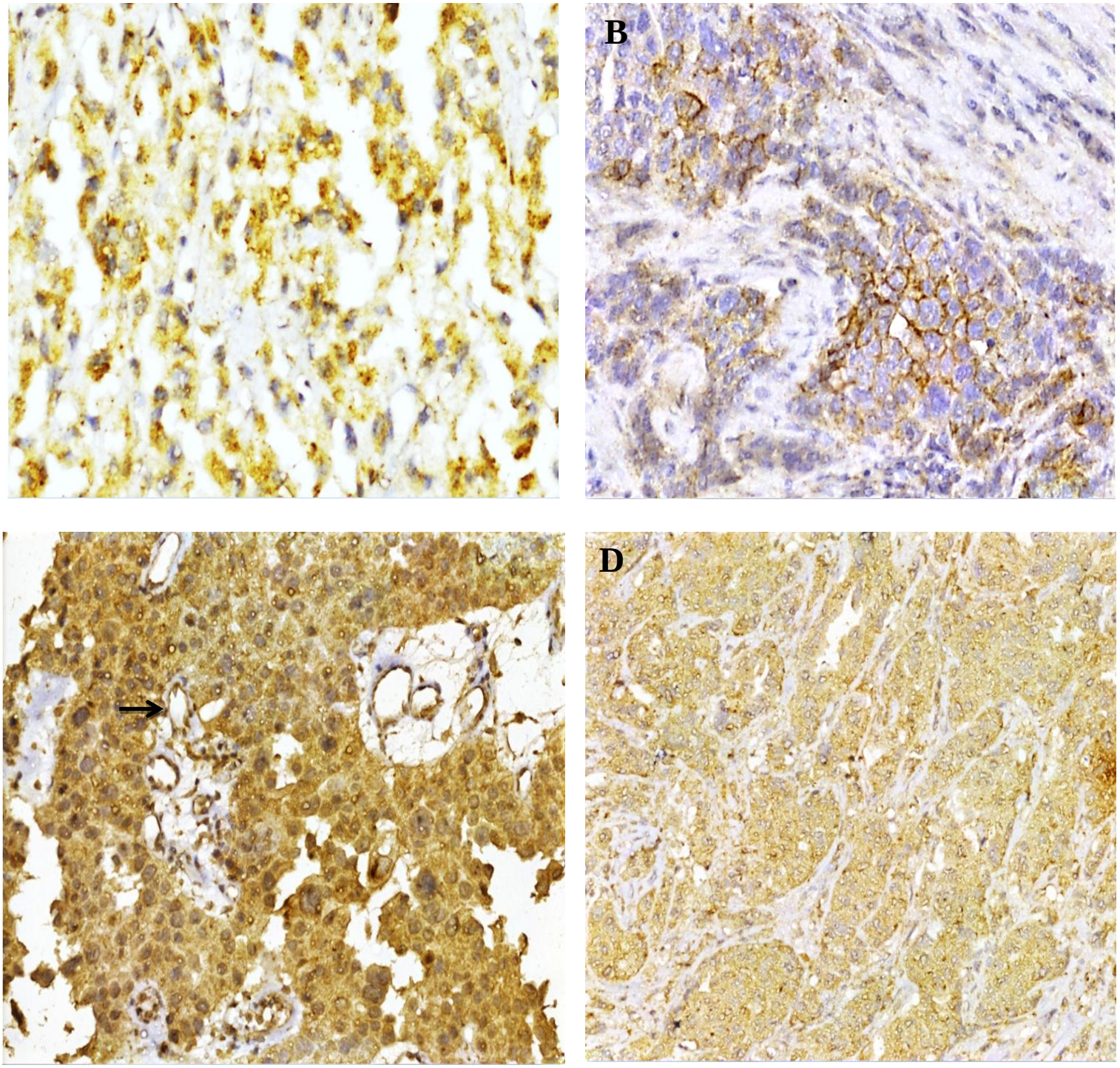
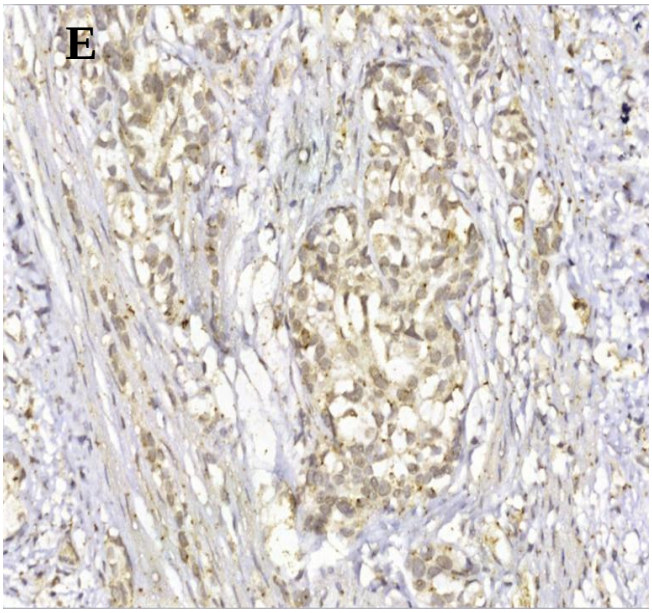
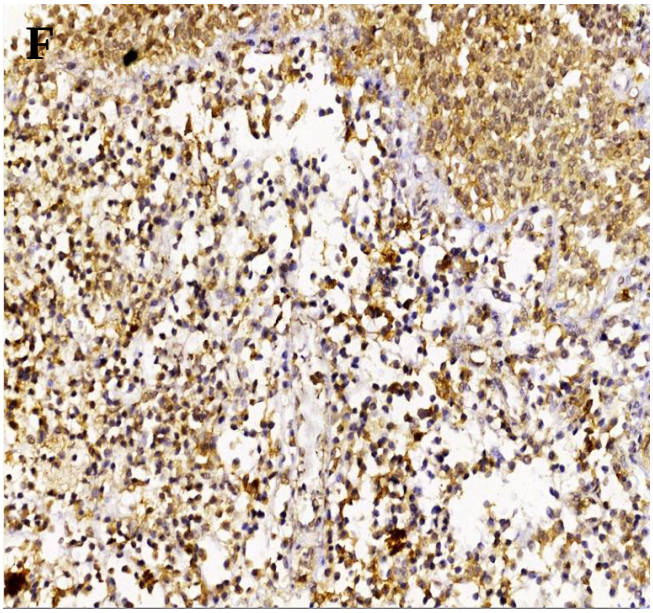


Figure (1): Immunohistochemical expression of TIM-3. **A**: Positive cytoplasmic expression in UC cells (original magnification (OM); x40). **B**: Positive membranous expression in UC cells (OM; x40). **C**: strong cytoplasmic TIM-3 staining intensity in UC cells, as well as TIM-3 expression by endothelial cells (arrow) (OM; x20). **D**: Intermediate cytoplasmic TIM-3 staining intensity in UC cells (OM; x20).





(Figure 1; continued): E: Negative/weak cytoplasmic TIM-3 staining intensity in UC cells (OM; x20).

F: TIM-3 positive tumor infiltrating mononuclear cells (OM; x20).

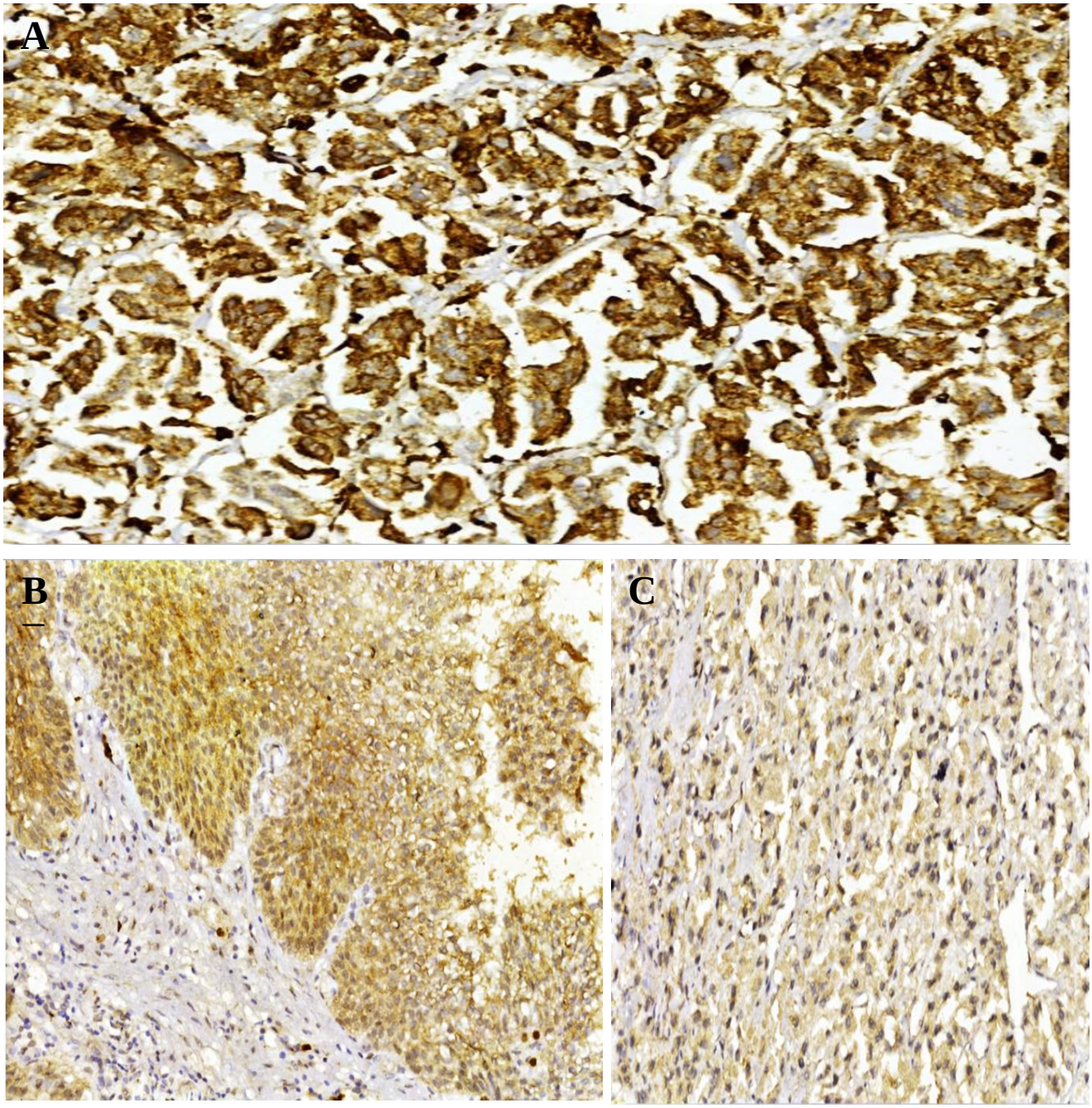


Figure (2): Immunohistochemical expression of CEACAM-1 in UC cells. **A**: Strong cytoplasmic & membranous CEACAM-1 staining intensity (OM; x20). **B**: Intermediate cytoplasmic CEACAM-1 staining intensity (OM; x10). **C**: Weak cytoplasmic CEACAM-1 staining intensity (OM; x10).

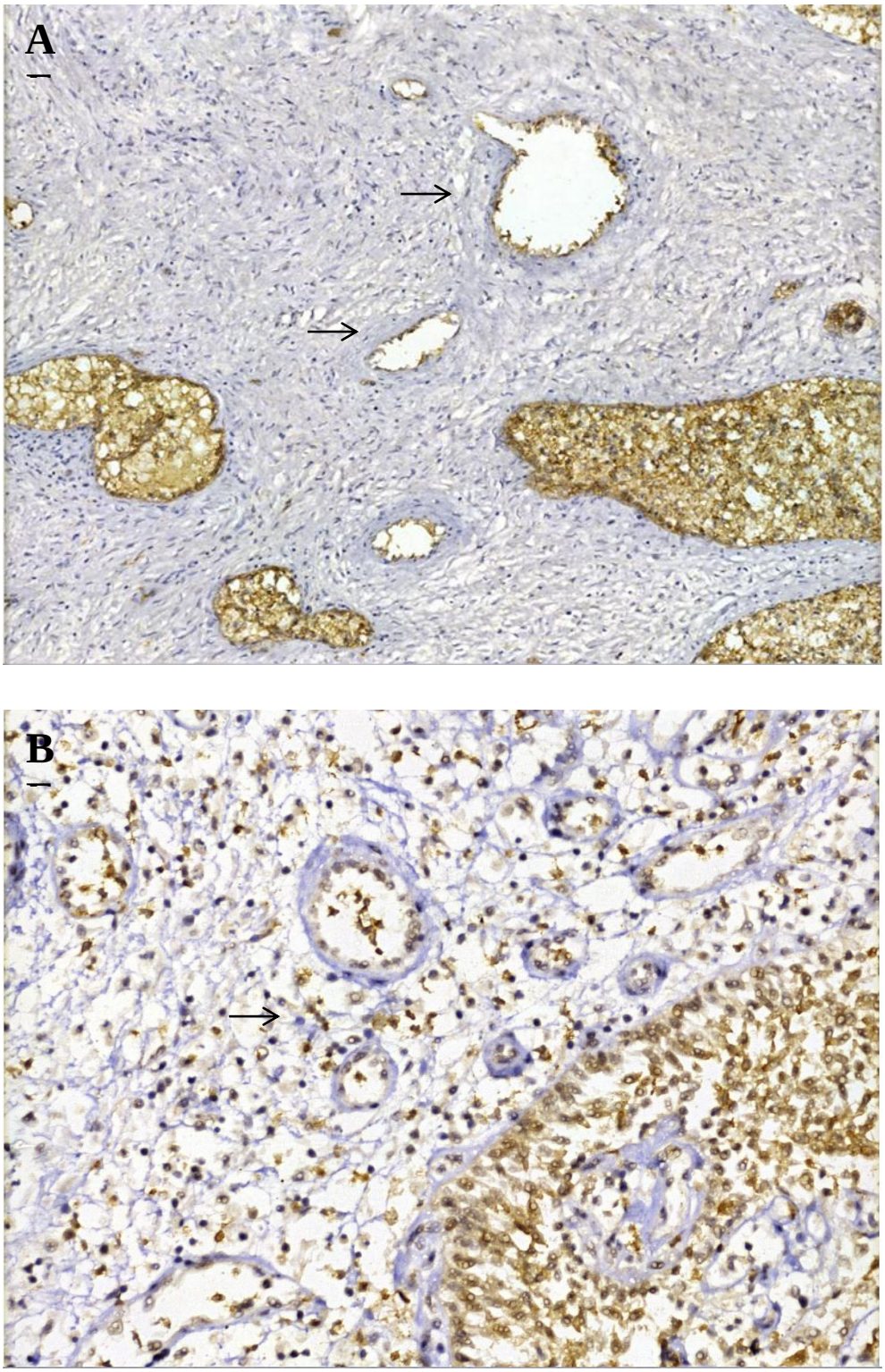


Figure (3): Invasive high grade urothelial carcinoma **A**: showing positive cytoplasmic CEACAM-1 expression, as well as CEACAM-1 expression by endothelial cells (arrow) (OM; x10). **B:** CEACAM-1 positive tumor infiltrating mononuclear cells (arrow) (OM; x20).

4. Discussion:

Bladder cancer ranks the 9th most frequently diagnosed cancer in the world, and the 13th in terms of deaths **(16).** Immune checkpoint inhibition is one of the most exciting and promising areas of research in cancer therapeutics. Targeting TIM-3 might be a promising approach for cancer immunotherapy. A recent study have highlighted that TIM-3 has an important role to play in T-cell exhaustion and correlates with the outcome of anti-PD-1 therapy **(17).**

The co-expression of CEACAM-1 is required for TIM-3 glycosylation and protein stability, and the inhibitory function of TIM-3 is compromised in the absence of CEACAM-1expression **(18).**

From this point of view, this research was concerned with studying the expression of both TIM-3 and CEACAM-1 in urothelial carcinoma of the urinary bladder in a group of Egyptian patients aiming that both of them could be target molecules in urothelial carcinoma therapy.

In accordance with Yang et al., 2015 who were the first to report TIM-3 expression in bladder urothelial carcinoma **(6)**, the present study revealed that immunohistochemical TIM-3 expression was detected in bladder cancer cells (100% of cases), TILs (93% of cases) and endothelial cells (91.7% of cases).

The high expression of TIM-3 by tumor cells in this study was more frequently associated with tumor necrosis. This could be explained by that TIM-3 expression in malignant cells may represent one of the various mechanisms used by the tumor cells to escape immune surveillance facilitating their onset, rapid growth and dissemination. As known, rapidly growing malignant tumors frequently encounter hypoxia and nutrient deprivation resulting in necrotic cell death in the core region of solid tumors.

It has been reported that the expression levels of TIM-3 were significantly correlated with advanced pathological grade and T stage **(6)**. Furthermore, a recent meta-analysis study concluded that TIM-3 expressions on tumor cells were significantly associated with poor overall survival in most solid cancers in human and related positively with nodal metastasis, tumor grade and PD-1 expression **(19).**

Our study revealed that high TIM-3 expression by tumor cells was more frequently associated with higher tumor grade, stage and nodal metastasis but these relations were statistically insignificant. This may be due to small sample size of both low grade tumors and radical cystectomy specimens in our study.

In line with our study, TIM-3 expressions in tumor cells have also been reported in several human malignancies including melanoma, gastric cancer, lung cancer, cervical cancer, and prostate cancer indicating that TIM-3 may not only suppress

anti-tumor immunity, but also directly promote cancer progress **(20)**.

In this study, TIM-3 expression by TILs was high in 63.3% of cases and showed significant correlation with TIM-3 expression by tumor cells indicating that TIM-3 may play a key role in the bladder UC related immune suppression. It has been shown that TIM-3 expression is elevated on circulating or tumor-infiltrating T cells from patients with other various tumors, and is associated with progression and poor prognosis **(21)**. Several studies also confirmed that TIM-3 expression in TILs is associated with severe T cell dysfunction in several types of cancers including renal cell carcinoma, gastric cancer, and NSCLC **(5)**.

In bladder cancer patients Zhang et al 2018 reported increased TIM-3 expression in NK cells with reduced cytotoxicity and TIM-3 blockade has allowed the NK cells to acquire higher cytotoxicity **(22)**. Another study has demonstrated that there was a trend toward a higher expression of TIM-3 in NK cells, CD4+ and CD8+ T cells in patients who were refractory to BCG compared to responders, suggesting a possible role in the disease aggressiveness **(23)**.

Regarding which is better, with unexplained mechanism TIM-3 expression on tumor cells has been reported to have greater prognostic value than its expression on TILs despite being mainly expressed on immune cells with lower expression in tumor cells **(19)**.

In the present study, TIM-3 expression by endothelial cells positively correlated with TIM-3 expression on TILs. Endothelial TIM-3 expression was also reported in melanoma **(24)**, B-cell lymphoma **(25)** and osteosarcoma **(26)** promoting their growth and metastasis.

Regarding CEACAM-1 expression, it was noted in 90% of the studied cases within cancer cells and endothelial cells. The endothelial expression was significantly related to tumor grade and muscle invasive group. Similarly, Oliveira-Ferrer et al., 2004 reported that all cases with invasive bladder tumors showed CEACAM-1-positive blood vessels in close association with the tumor cell groups. However, they stated that CEACAM-1 expression appears to be downregulated in bladder cancer cells, while concurrently upregulated in endothelial cells of tumoral adjacent blood vessels **(27)**.

This conflict regarding CEACAM-1 expression by cancer cells whether up or downregulated could be explained by the fact that CEACAM-1 has many isoforms as well as the different antibody clones used and different staining conditions. Indeed, many results have demonstrated an isoform-specific functionality of CEACAM-1, thus increasing the complexity of this system **(28).**

Our study revealed that CEACAM-1 endothelial expression was significantly

related to presence of tumor necrosis and increased associated inflammation. It has been recognized that inflammation and necrosis promote tumor growth; and the inflammatory pathways are important for angiogenesis, stromagenesis and epithelial proliferation promotion **(29)**. In addition, endothelial CEACAM-1 expression is believed to stimulate vascular endothelial growth factor and fibroblast growth factor dependent proangiogenic activities and thus, promoting angiogenesis **(30)**.

Furthermore, our study revealed high significant relations between tumor subtypes and expression of CEACAM-1 by endothelial cells as all negative cases (n=6) were of papillary differentiation. This could be explained by that our all studied cases with papillary differentiation were non-muscle invasive. Thus, from our above significant correlations of endothelial CEACAM-1 expression, the latter could be a marker of tumor progression and invasiveness.

However, no significant correlation has been found in our study between endothelial CEACAM-1 expression and lymphovascular invasion. This could be explained by the fact that CEACAM-1 has another function in endothelial cells besides promoting angiogenesis which is regulation of vascular integrity and permeability **(31).**

Our study showed that CEACAM-1 expression appeared in 96% of cases within TILs. Many studies have demonstrated that CEACAM-1 expressed on the surface of effector immune cells is considered to be a crucial molecule in the down-regulation of immune responses and may inhibit cytotoxicity and attenuate antitumor immunity in natural killer cells. Moreover, antitumor effects have been enhanced by using monoclonal antibodies to block the inhibitory CEACAM-1 pathway in malignant melanoma and lung cancer **(32)**.

Also, significant positive correlation was found between CEACAM-1 expression by both TILs and tumor cells. Keeping with our findings, many studies have demonstrated that homophilic interactions of CEACAM-1, occurring between CEACAM-1 positive TILs and CEACAM-1 positive tumor cells, may inhibit multiple effector functions of TILs thus protecting tumor cells from immune attacks **(33, 34, 35)**. Similar findings have been reported in gliomas **(32)**. Thus, CEACAM1 may be a promising target candidate for urothelial carcinoma immunotherapies.

An apparently paradox finding in the present study is that the high expression of CEACAM-1 in TILs was more frequently associated with absent vascular emboli. It worth mentioning that CEACAM-1 can function as a tumor suppressor as CEACAM1-L isoforms can transduce inhibitory signals **(36)**. Indeed, CEACAM-1 works together with specific signaling factors, proteins and

receptors, dependent on the various contexts in which it occurs, therefore, its role should be interpreted separately in each of these different cell types, tissues and pathological conditions **(30)**.

In this study, CEACAM-1 expression in TILs was significantly positively correlated with TIM-3 expression in both TILs and tumor cells. Also, positive CEACAM-1 endothelial cell expression was more frequently associated with high TIM-3 expression by tumor cells (P value; 0.029). As mentioned before, CEACAM-1 serves as a heterophilic ligand for TIM-3 which is required for its ability to mediate T-cell exhaustion, and this interaction is crucial in regulating anti-tumor immunity **(7).** In colon cancer, **Zhang et al., 2017** have reported that co-expression of TIM-3 and CEACAM1 on TILs can promote T cell exhaustion **(9)**.

However, a recent study has demonstrated that CEACAM1 inhibits T cell activation, but without any evidence that this receptor has a role in TIM-3 function. Also, co-transfection experiments in that study did not indicate that CEACAM1 promotes surface expression of TIM-3 **(37)**.

5. Conclusion and Recommendations:

The high co-expression of CEACAM-1 and TIM-3 in TILs may play a key role in the bladder urothelial carcinoma related immune suppression. Moreover, the multiple significant correlations found between different TIM-3 expressions and CEACAM-1 expression**s** support the data describing the latter as heterophilic ligand for the former. In addition, the aberrant expression of TIM-3 within tumor cells and its relation to necrosis may indicate a role for TIM-3 in urothelial cancer development. Added to that, endothelial CEACAM-1 expression could be a marker of tumor progression and invasiveness as it was expressed in muscle invasive urothelial cancers and absent in non-muscle invasive caners with papillary differentiation and in most low grade tumors in this study.

New treatment targeting TIM-3 and CEACAM-1 could offer a breakthrough in cancer treatment and improve patient outcomes. Future studies with larger sample sizes are recommended to more explore the function of TIM-3 and CEACAM-1 and other immune checkpoint inhibitors in bladder urothelial carcinoma that could be a promising immunotherapeutic targets.

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