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RESEARCH ARTICLE

Role of YAP1 and tumor-associated macrophages in urothelial carcinoma of urinary bladder: Histopathological and immunohistochemical studies

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ABSTRACT

Background: Urothelial Carcinoma (UC) of the urinary bladder is the most predominant histologic pattern of bladder cancer. Studying the prognostic aspects of UC through spotting the light on Yes-associated protein 1 (YAP1) together with TAMs may help to know their role in the prognosis of UC. **Aim**: Our study aims at reviewing the immunohistochemical reactivity of YAP1 and TAMs marker CD68 in UC. Additionally, to evaluate the relation between YAP1 and CD68 with the available clinicopathologic parameters in UC and with each other. **Material and Methods:** Eighty-two paraffin blocks of UC were collected retrospectively and included 62 trans-urethral resection of bladder tumor specimens and 20 specimens from radical cystectomies. All were stained with Hematoxylin & Eosin, YAP1, and CD68. **Results:** YAP1 expression was related significantly to large-sized tumors, histopathologic subtypes of UC (conventional UC versus plasmacytoid type), high tumor grade, advanced pT (size and extent of the main tumor) and N (number of lymph nodes that have cancer) stage, and perineural invasion (PNI). $CD68^{+}$ Tumor- associated macrophages (TAMs) mean count was significantly related to large tumor size. The histopathologic types of UC (papillary non-invasive versus invasive), histopathologic subtypes of UC (conventional UC versus UC with squamous differentiation and plasmacytoid type), tumor grade, advanced pT stage, and PNI. **Conclusion:** High YAP1 expression and $CD68^{+}$ TAMs count was significantly related to adverse pathological factors, namely large-sized tumors, tumors of high grade, and advanced pT stage. Their expression in UC could be used in identifying patients at increased risk of tumor invasion and progression.

Keywords: TAMs, Urinary Bladder, Urothelial Carcinoma, YAP-1

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INTRODUCTION

Urinary bladder carcinoma (BC) is the 10th prevalent malignancy across the world and the 3rd in Egypt. Its prevalence is gradually increasing globally, particularly in industrialized nations (Sung et al., 2021). BC is a heterogeneous tumor characterized by having a variety of pathologic factors affecting the prognosis. However, their cardinal mechanisms of action have yet to be fully discovered (Russo et al., 2023). Despite the treatments of BC, the overall survival of patients with advanced BC is still unsatisfactory (Leblond et al., 2021).

Up to the present time, the well-settled and commonly used clinicopathological factors to predict BCs prognosis are TNM staging and tumor grade. However, prognosis and response to treatment of BC patients with the same grade and stage often differ greatly (Larrinaga et al., 2021). Thus, several studies on BC have tried to discover novel biomarkers which would serve as reliable predictive tools for bladder carcinoma, especially in that era where target therapy is developing.

The hippo signaling pathway is a recently revealed tumor suppressor signal pathway that works on regulating cellular division and it encloses a single oncogene beside multiple tumor-suppressing genes (Jia et al., 2019). Yes-associated protein 1 (YAP1) is considered a main transcription moderator in fostering tumor formation and it directs the action of many pro-oncogenes such as CTGF, KRAS, and Wnt/β-catenin (Zheng and Pan, 2019).

YAP1 over reactivity amplifies the dissemination of tumor cells by encouraging intravascular

motility thus facilitating the re-entry of tumor cells into the systemic circulation and acting as a contributing factor in the resistance to many relevant therapeutic agents (Benjamin et al., 2020). Moreover, YAP1 was found to participate in immune evasion adopted by malignant cells in many cancers by binding directly to the enhancer and stimulating the expression of the immune checkpoint protein, PDL-1 gene (Szulzewsky et al., 2021). Recently, YAP1 is documented to be highly expressed in various cancer types encompassing liver (Jiang et al., 2022), colon (Ma et al., 2017), prostate (Jiang et al., 2017), and breast cancers (Shao et al., 2014). However, to date, the study of YAP1 expression and its clinicopathologic prognostic implication in BC remains substantially limited.

The hippo signaling pathway is a major contributor to the immune response, especially in the functional synchronization of tumor-associated macrophages (TAMs). As YAP1 is the basic moderator of the Hippo pathway, it not only aids the progress of tumors but works as a regulator balancing the relation between immune cells and malignant cells as well (Feng et al., 2018).

TAMs are considered one of the most presented immune cells infiltrating solid tumors (Fu et el., 2018). Despite their probable dual action as pro- or anti-tumoral, numerous studies reported that TAMs recruitment is strongly accompanied by bad prognosis in various tumor types (Zhang et al., 2021). CD68 is known to be a pan-macrophage marker expressed by both M1 and M2 subsets. $CD68^+$ macrophages in TME obtain immunosuppressive features and inhibit $CD8^+$ cytotoxic T cells in tumors (Kitamura et al., 2015).

$CD68^+$ TAMs high expression in tumor interstitium frequently correlates with higher histological grade, nodal invasion, and distant metastasis together with other features that characterize tumor progress and aggressiveness (Ni et al., 2015). Escalating evidence has reported that CD68 is a promising prognostic as well as a diagnostic marker in cancers (Zhang et al., 2022).

$CD68^+$TAMs have a crucial function in tumor immunity and affect the overall prognosis. Thus, it is crucial to discover the impact of YAP1 on them to provide new visions into its effective implication as a new target for immunotherapy in BC.

This work aims to review the immunohistochemical expression of YAP1 and $CD68^+$TAMs in urothelial bladder carcinoma and to assess the relation between these two markers and the available clinicopathological factors as well as the correlation between the expression of both markers in the studied urothelial carcinoma cases.

MATERIALS AND METHODS

Study design and data collection

This is a cross-sectional study that was carried out on 82 selected specimens diagnosed previously as primary urothelial carcinoma of the urinary bladder (62 TURBT specimens and 20 radical cystectomies with lymphadenectomy specimens). The specimens were gathered from the archive of the Pathology department, Faculty of Medicine, Tanta University, Egypt as well as from the newly received specimens in the department during the period of the study from December 2022 to December 2023. The current study was conducted after obtaining the approval from the institutional research ethics committee at Faculty of Medicine, Tanta University, Egypt (Code #36126/11/22).

before initiating data collection. All cases included in this work have complete clinicopathologic data and sufficient tumor tissue, while recurrent cases are those who received neoadjuvant chemo or radiotherapy and tumors with extensive necrosis were excluded from the study.

Histopathologic evaluation

Five µm sections were serially cut and mounted on glass slides to be stained with hematoxylin & eosin and were examined for confirming the histopathologic diagnosis and assess the histological tumor type according to the WHO classification of urothelial *carcinoma* (Netto et al., 2022), tumor grade referring to the two tired system of grading (Moch et al., 2016), where pTa and pT1 tumors non-muscle-invasive bladder carcinoma (NMIBC) were graded according to their morphological features into low-grade and high-grade, while all muscle-invasive bladder carcinoma (MIBC) are

considered as high-grade tumors, pT stage according to AJCC/TNM staging system of UC of UB (Amin et al., 2017) and regional lymph node invasion (N stage) only in cystectomy with lymphadenectomy specimens as well as to detect lymphovascular and perineural invasion, associated UCIS and bilharzial infestation (based on detection of *Schistosoma* eggs in tumor tissues or adjacent non neoplastic tissue).

Immunohistochemical processing and staining

Paraffin embedded tissue blocks are cut into 5-µm thick sections and placed on positively charged slides. These slides were then left to dry at 37℃ for 30 min. After that, deparaffinization and antigen retrieval took place in a Dako PT Link unit. pH 6.0 EnVisionTM FLEX retrieval target solutions were utilized for 20 min. Immunohistochemistry of both primary antibodies had been done on Dako Auto-stainer Link 48. The Antibodies included in our study were YAP1 rabbit polyclonal antibody (CloneARC53479, dilution1:500, ABclonal- USA, California) and CD68 monoclonal antibody (clone PG-M1, dilution 1:100, Dako). The treatment of the slides was done by using a peroxidase blocking reagent for 5 min. to be incubated in the primary antibodies for 20-30 min. Afterwards, slides were placed for 20 min. into horseradish peroxidase (HRP) polymer reagent, and then counter-stained with hematoxylin.

Evaluation of YAP1 immunostaining

Brownish nuclear staining of YAP1 in the malignant epithelial cells was considered positive IHC staining. Semiquantitative evaluation was done using the percentage of stained cells and staining intensity, where the staining intensity was scored as follows: 0: Negative, 1: Weak, 2: Moderate and 3: Strong. The percentage of stained cells was scored as; positive cells are 0: 0-5%, 1: positive cells are 6-25%, 2: positive cells are 26-50%, 3: positive cells are 51-75%and 4: positive cells are 76-100% of tumor cells. Finally, YAP1 total score was evaluated by "multiplying" the intensity score by the percentage score to get a final score ranging from (0- 12). Scores <6 define low YAP1 expression and scores ≥ 6 mean high YAP1 expression (Ibrahim et al., 2022).

Quantitative scoring of $CD68^+$ TAMs

Image analysis software Fiji (ImageJ bundled with plugins) was used to count the $CD68^+$ TAMs, where three hot spots were selected and captured for image analysis. Afterward, the $CD68^+$ TAMs were counted manually at high power magnification (x400) using the plug-in "cell counter" in the ImageJ software, then the mean count for three hot spots was calculated for each case. The median value for $CD68^+$ TAMs was taken as a cutoff point to categorize the studied cases into two groups: High $CD68^+$ means count group (> median value) and low $CD68^+$ means count group (≤ median value) (Jamiyan et al., 2020).

Statistical analysis

The results were analyzed statistically using SPSS. Categorical variables were represented as frequencies (numbers of cases) and percentages, while median or mean ± standard deviation (SD) was represented for continuous variables. The relationship between clinicopathological factors and the expression of YAP1 and CD68 was performed using the Chi-square (X^2) test as a test of significance. Fisher's exact test or Monte Carlo was utilized if ≥1 cells show an expected frequency of ≤5. The correlation between biomarkers expression was done via Pearson correlation (r). P-values <0.05 were regarded as statistically significant.

RESULTS

Clinicopathologic characteristics

The mean age was 63±10.98 years, 73.2% of studied cases were males. The size of the tumors was assessed by pelviabdominal U/S and manual measurements during gross examination (radical cystectomy specimens), it ranged 1-8 cm with a mean size of 4±1.735 cm. The multicentricity of the tumor was assessed by pelviabdominal ultrasound and during gross examination (radical cystectomy specimens). It was found that 70.7% of cases presented with a single tumor mass. According to the WHO 2022 classification of UC of UB, the studied cases were 14 non-invasive papillary UC (17.1%) and 68 invasive UC (82.9%). The clinicopathologic data of the studied cases are shown in Table 1.

Table 1. The clinicopathologic data studied urothelial carcinoma (UC) cases.

Clinicopathological data of studied UC cases	Number (%)
Age (years)	
Mean±SD	6310.98
≤60	35 (42.7)
>60	47 (57.3)
Gender	
Male	60 (73.2)
Female	22 (26.8)
Tumor size	
≤ 3cm	50 (61)
> 3 cm	32 (39)
Tumor Multicentricity	
Single	58 (70.7)
Multiple	24 (29.3)
Histopathological type	
Non-invasive papillary UC	14 (17.1)
Invasive UC	68 (82.9)
Conventional UC	34 (41.5)
UC with squamous differentiation	16 (19.5)
UC with glandular differentiation	8 (9.8)
Plasmacytoid subtype	4 (9.4)
Micropapillary subtype	2 (2.4)
Nested subtype	2 (2.4)
Sarcomatoid subtype	2 (2.4)
Tumor grade	
Low grade	15 (18.3)
Non-invasive papillary UC	10 (12.2)
Invasive UC	5 (6.1)
High-grade	67 (81.7)
Non-invasive papillary UC	4 (4.9)
Invasive UC, non-papillary	63 (76.8)
Conventional UC	29 (35.4)
UC with squamous differentiation	16 (19.5)
UC with glandular differentiation	8 (9.8)
Plasmacytoid subtype	4 (4.9)
Micropapillary subtype	2 (2.4)
Nested subtype	2 (2.4)
Sarcomatoid subtype	2 (2.4)
pT staging of UC (TURBT specimens)	
pTa	14 (22.6)
PT1	8 (12.9)
pT2a	40 (64.5)
pT staging of UC (cystectomy specimens)	
pT2b	4 (20)
PT3a	8 (40)
pT3b	2 (10)
PT4a	6 (30)
N staging of radical cystectomy specimens	
N0	10 (50)
N1	7 (35)
N2	3 (15)
Lympho-vascular invasion (LVI)	
Absent	64 (78)
Present	18 (22)
Perineural invasion (PNI)	
Absent	71 (86.6)
Present	11 (13.4)
Concomitant UCIS	
Absent	72 (87.8)
Present	10 (12.2)
Associated Schistosomiasis	
Absent	61 (74.4)
Present	21 (25.6)

YAP 1 immunohistochemical results

YAP1 positive reactivity was defined as brownish staining in the nuclei of tumor cells, where high expression of YAP1 was detected in 47/82 (57.3%) of included cases. Representative images are displayed in Figure 1. High YAP1 expression was frequently detected in UC measured > 3 cm, where the relation between YAP1 and tumor size was statistically significant (p-value=0.014). Similarly, a significant statistical relation had been found between YAP1 and tumor grade (p-value=0.001) with the majority of high-grade UC (65.7%) showing high YAP1 expression.

On analyzing the difference in YAP1 expression between the conventional invasive UC and the other histological subtypes, a significant statistical difference was detected between conventional UC and plasmacytoid type (p-value=0.008). However, no statistically significant difference was detected between YAP1 expressions in conventional UC versus the other subtypes as demonstrated by the results in Table 2. There was a significant statistical relation between the pT stage and YAP1 expression (p-value= 0.001). Besides, the relation between YAP1 expression and the extent of invasion in MI and beyond UC cases was further analyzed and exhibited a significant difference (p-value= 0.001).

A significant statistical relation was found between YAP1 expression and N stage of UC in radical cystectomy cases (p-value= 0.005). There was no significant statistical relation between YAP1 expression and LVI (p-value=0.148). However, the relation between YAP1 expression and PNI was statistically significant (p-value=0.015). Regarding the associated UCIS and associated schistosomiasis, neither had shown a significant statistical relation with YAP1 expression. The relation between the immunohistochemical expression of YAP1 and clinicopathological parameters is illustrated in Table 3.

CD68 + immunohistochemical results

$CD68^+$TAMs in peritumoral and intratumoral compartments were counted, then $CD68^+$TAMs mean count was evaluated for each case and the median value for all included cases mean counts was (31). This median value was taken as a cutoff point to categorize studied UC cases into two groups; *High $CD68^+$TAMs mean count group* with $CD68^+$TAMs mean count >31, and low $CD68^+$ mean count group that includes cases with $CD68^+$ TAMs mean count ≤ 31. Accordingly, 48.8% of studied UC cases showed a high $CD68^+$ mean count and 51.2% showed a low $CD68^+$ mean count. The representative images are displayed in Figure 2.

The relation between $CD68^+$TAMs mean count and clinicopathological factors is demonstrated in Table 4. There were no statistically significant relations between $CD68^+$TAMs mean count and age of patients age nor their gender. However, Tumor size showed a significant relation to $CD68^+$TAMs mean count (p-value=0.011). All Low-grade UCs showed low $CD68^+$ TAMs mean count, while in high-grade UCs, 59.7% showed a high mean count. A significant statistical relationship was found between the $CD68^+$ which means count and tumor grade (p-value < 0.001).

All non-invasive papillary UC showed a low $CD68^+$ mean count, whereas in invasive UC, 58.8% showed a high $CD68^+$ mean count and 42.2% showed a low count**.** There was a significant statistical relationship between $CD68^+$ TAMs mean count and the histopathologic types of UC (p-value < 0.001). On testing the difference of $CD68^+$ TAMs mean count between the conventional invasive UC and other histological subtypes; a significant statistical difference was detected with UC with squamous differentiation (p-value< 0.001) and with plasmacytoid subtypes (p-value=0.003). However, no statistically significant difference of $CD68^+$ TAMs mean count between conventional UC and other subtypes was found.

The relation between $CD68^+$ TAMs mean count and the pT stage revealed a significant statistical relation (p-value < 0.001). However, no significant statistical difference was found between CD68+ TAMs mean count in relation to the extent of invasion in MI and beyond UC. In radical cystectomy specimens, analyzing the relation between $CD68^+$ means count, and N stage doesn't reach statistical significance.

Analyzing the correlation between YAP1 and CD68+ TAMs mean count revealed a statistically

Table 2. Difference of YAP1 expression between the conventional invasive UC and other histological subtypes

YAP1 expression N (%)	Conventional UC	UC with squamous differentiation	UC with glandular differentiation	Plasmacytoid UC	Micropapillary UC	Nested UC	Sarcomatoid UC
Low 26 (38.2)	11 (32.4)	5 (31.3)	4 (50)	4 (100)	0 (0)	0 (0)	2 (100)
High 42 (61.8)	23 (67.6)	11 (68.8)	4 (50)	0 (0)	2 (100)	2 (100)	0 (0)
X^2		0.006	0.878	6.855	0.932	0.932	3.747
P- value		0.938	0.348	0.008*	0.334	0.334	0.05

UC: urothelial carcinoma, *: p-value <0.05, x^2: Chi square test

Table 3. The relation between YAP1 expression and clinicopathological parameters.

Parameter	YAP1 expression		P value
	Low	High	
Age			
≤ 60	15 (42.9)	20 (57.1)	0.978 X2
> 60	20 (42.6)	27 (57.4)	
Gender			
Male	26 (43.3)	34 (56.7)	0.844 X2
Female	9 (40.9)	13 (59.1)	
Tumor size			
> 3 cm	16 (32)	34 (68)	0.014* X2
≤ 3 cm	19 (59.4)	13 (40.6)	
Multicentricity			0.905 X2
Single	25 (43.1)	33 (56.9)	
Multicentric	10 (41.7)	14 (58.3)	
Histopathologic type			0.070 X2
Noninvasive UC	9 (64.3)	5 (35.7)	
Invasive UC	26 (38.2)	42 (61.8)	
Tumor Grade			
Low	12 (80)	3 (20)	0.001* X2
High	23 (24.3)	44 (65.7)	
pTstage			0.001* X2
NMI (Ta, T1)	16 (72.7)	6 (27.3)	
MI (T2, T3, T4a)	19 (31.7)	41 (68.3)	
Extent of invasion			
pT2a	12 (30)	28 (70)	0.025* MC
pT2b	4 (100)	0 (0)	
pT3a	1 (12.5)	7 (87.5)	
pT3b	0 (0)	2 (100)	
pT4a	2 (33.3)	4 (66.7)	
N stage			
N0	7 (70)	3 (30)	0.005* MC
N1	0 (0)	7 (100)	
N2	0 (0)	3 (100)	
LVI			0.148 X2
Absent	30 (46.9)	34 (53.1)	
present	5 (27.8)	13 (72.2)	
PNI			
Absent	34 (47.9)	37 (52.1)	0.015* X2
Present	1 (9.1)	10 (90.9)	
UCIS			
Without	30 (41.7)	42 (58.3)	0.618 X2
With	5 (50)	5 (50)	
Associated schistosomiasis			
Absent	26 (43.3)	34 (56.7)	0.844 X2
Present	9 (40.9)	13 (59.1)	

UC: urothelial carcinoma, *: p-value <0.05, x^2: Chi square test, MC: Monte Carlo test

Table 4. The relation between CD68+TAMs mean count and clinicopathological parameters.

Parameter	CD68+ TAMs mean count		P-value
	Low No. (%)	High No. (%)	
Age			
≤ 60 (n=35)	22 (62.9)	13 (37.1)	0.069 X2
> 60 (n=47)	20 (42.6)	27 (57.4)	
Gender			
Male (n=60)	29 (48.3)	31 (51.7)	0.388 X2
Female (n=22)	13 (59.1)	9 (40.9)	
Tumor size			
> 3 cm (n=50)	20 (40)	30 (60)	0.011* X2
≤ 3 cm (n= 32)	22 (68.8)	10 (31.2)	
Multicentricity			0.731 X2
Single (n=58)	29 (50)	29 (50)	
Multicentric (n= 24)	13 (54.2)	11(45.8)	
Histopathologic type			<0.001*X2
Non-invasive UC	14 (100)	0 (0)	
Invasive UC	28 (42.2)	40 (58.8)	
Tumor Grade			
Low (n=15)	15 (100)	0 (0)	<0.001*X2
High (n=67)	27 (40.3)	40 (59.7)	
pTstage			<0.001*X2
NMI (Ta, T1) (n=22)	21 (95.5)	1 (4.5)	
MI (T2, T3, T4a) (n=60)	21 (35)	39 (65)	
Extent of invasion			
pT2a (n=40)	14 (35)	26 (65)	0.538 MC
pT2b (n=4)	2 (50)	2(50)	
pT3a (n=8)	1 (12.5)	7 (87.5)	
pT3b (n=2)	1 (50)	1 (50)	
pT4a (n=6)	1 (16.7)	5 (83.3)	
N stage			
N0 (n=10)	4 (40)	6 (60)	0.269 MC
N1 (n=7)	1 (14.3)	6 (85.7)	
N2 (n=3)	0 (0)	3 (100)	
LVI			0.086 X2
Absent (n=64)	36 (56.3)	28 (43.7)	
Present (n=18)	6 (33.3)	12 (66.7)	
PNI			
Absent (n=71)	40 (56.3)	31 (43.7)	0.018*X2
Present (n=11)	2 (18.2)	9 (81.8)	
UCIS			
Without (n=72)	39 (54.2)	33 (45.8)	0.152X2
With (n=10)	3 (30)	7(70)	
Associated schistosomiasis			
Absent (n=60)	31 (51.7)	29 (48.3)	0.894 X2
Present (n=22)	11 (50)	11 (50)	

UC: urothelial carcinoma, *: p-value <0.05, x^2: Chi square test, MC: Monte Carlo test.

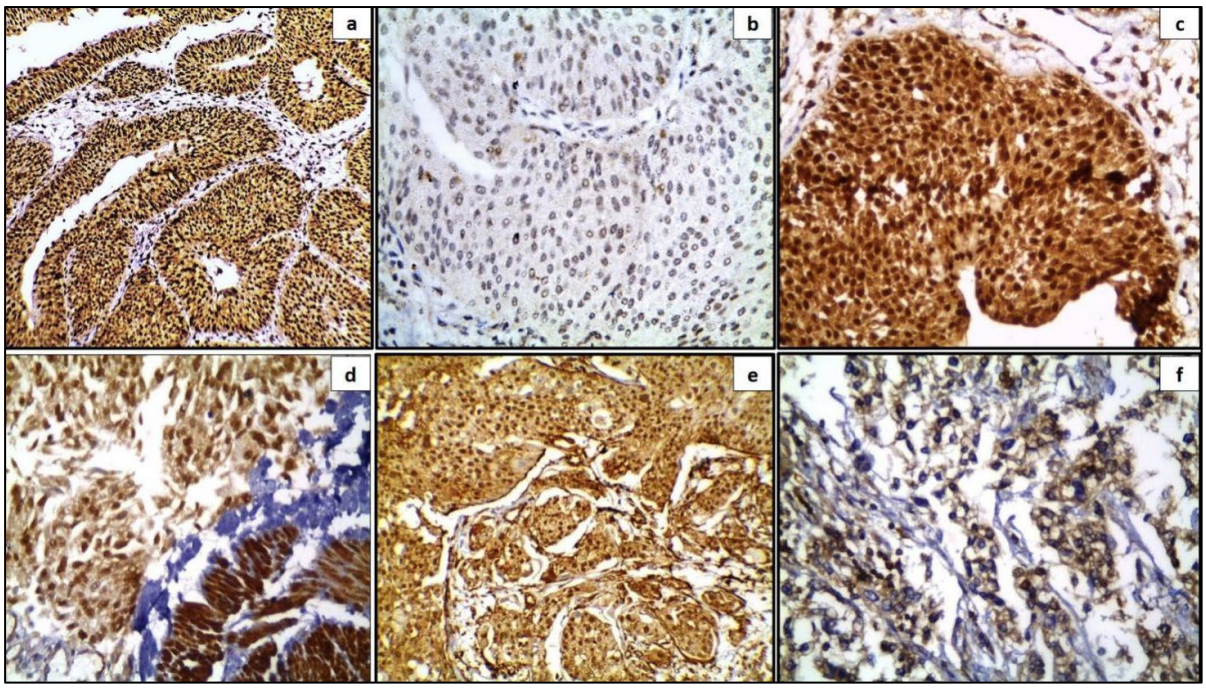

Figure 1. Yes-associated protein 1 (YAP1) immunohistochemical expression in urothelial carcinoma specimens. (a) Non-invasive papillary urothelial carcinoma, low-grade showing high nuclear expression of YAP1 (IHC ×200). (b) Non-invasive papillary urothelial carcinoma, low-grade showing low nuclear expression of YAP1 (IHC × 400). (c) Non-invasive papillary urothelial carcinoma, high-grade showing high nuclear expression of YAP1 (IHC × 400). (d) Conventional invasive urothelial carcinoma (Muscle invasive) showing high nuclear expression of YAP1 (IHC × 400). N.B smooth muscle is internal control. (e) Invasive urothelial carcinoma with squamous differentiation showing high nuclear expression of YAP1 (IHC × 200). (f) Sarcomatoid urothelial carcinoma showing low nuclear expression of YAP1 (IHC × 400).

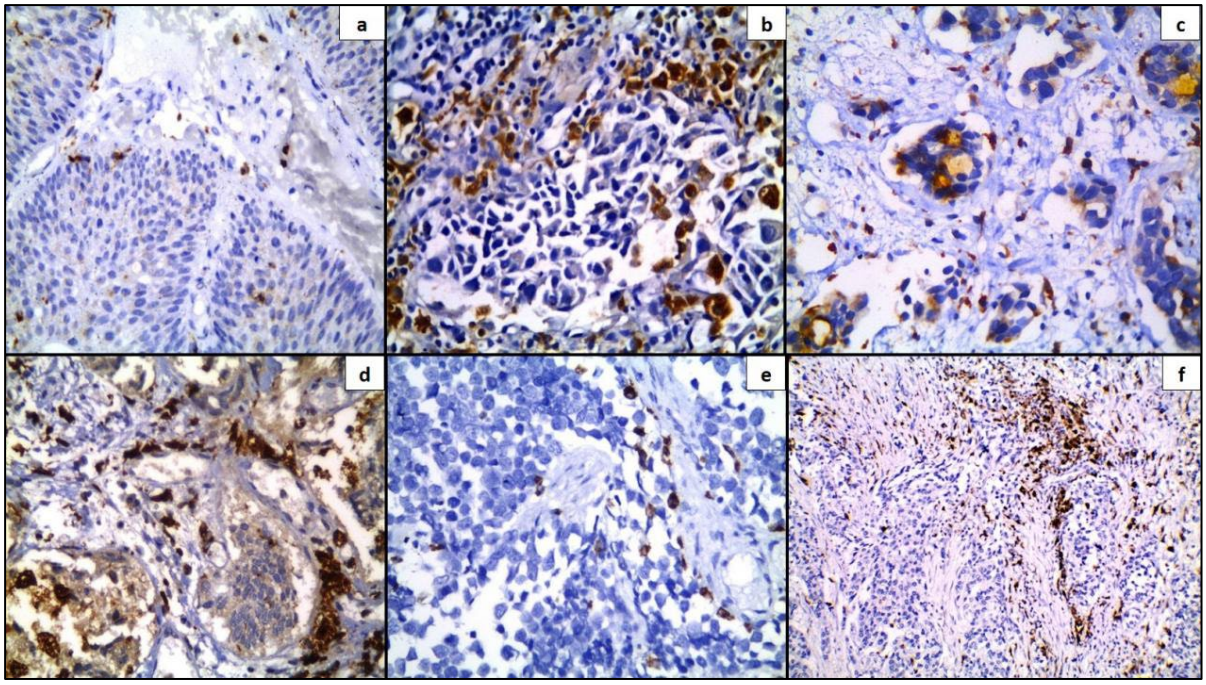

Figure 2. CD68^{+}TAMs immunohistochemical expression in UC studied cases. (a) Non-invasive papillary urothelial carcinoma, low-grade showing low CD68+ TAMs mean count (IHC ×400). (b) Conventional invasive urothelial carcinoma showing high CD68^{+} TAMs mean count (IHC × 400). (c) Invasive urothelial carcinoma with glandular differentiation showing high CD68^{+} TAMs mean count (IHC × 400). (d) Invasive nested urothelial carcinoma showing high CD68^{+} TAMs mean count (IHC × 400). (e) Invasive plasmacytoid differentiation urothelial carcinoma showing low CD68^{+} TAMs mean count (IHC × 200). (f) Invasive sarcomatoid urothelial carcinoma showing high CD68^{+} TAMs mean count (IHC×200)

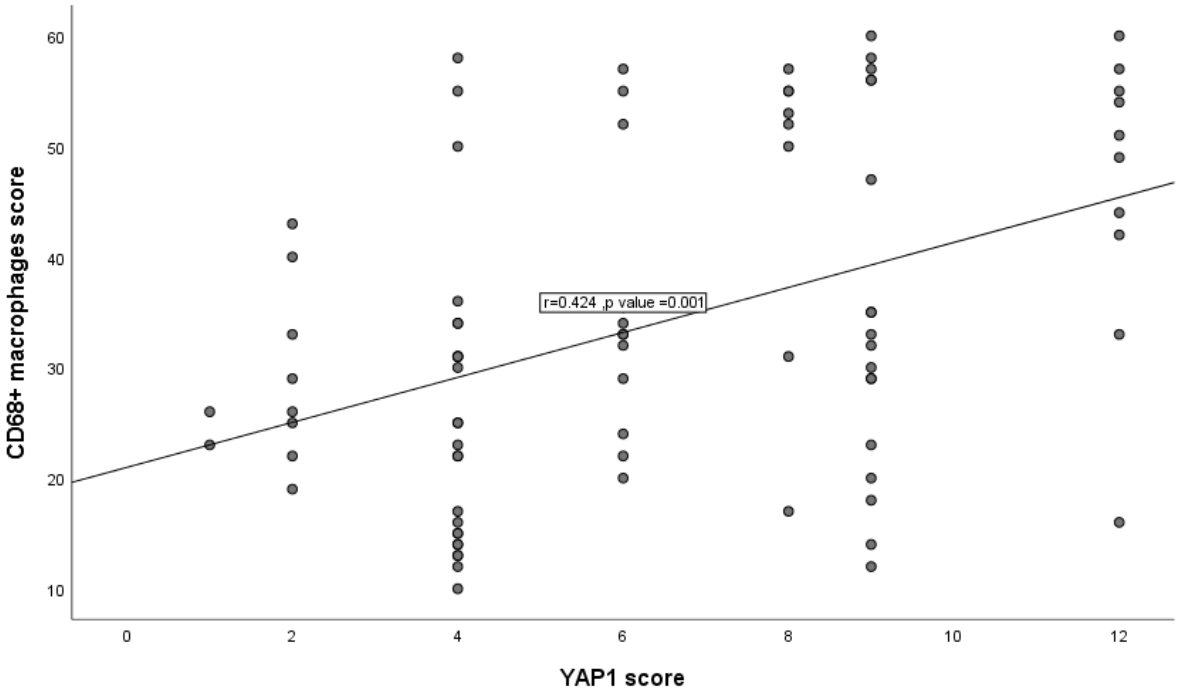

Figure 3. Scatterplot showing a statistically significant positive correlation between both markers (YAP1 and CD68$^+$ TAMs). TAMs: Tumor-associated macrophages, r: Pearson correlation coefficient, *: p-value < 0.05

significant positive correlation between both markers (r=0.424, p-value=0.001). Increased YAP1 expression was correlated with increased CD68$^+$ macrophage infiltration. A scatterplot summarizes the results (Figure 3).

DISCUSSION

Bladder cancer is considered one of the most frequently diagnosed and lethal human malignancies, with over 200,000 mortalities worldwide in 2023 (Chen et al., 2024). In Egypt, BC accounts for 30% of all malignancies, representing the main urinary system malignancy (Abulkhair et al., 2021). Based on the latest GLOBOCAN data, BC incidence is 3% accounting for 2% of all cancer deaths (Wéber et al., 2024).

YAP1 biomarker, a transcriptional co-activator affiliated to the Hippo pathway of tumor suppression (Elfadadny et al., 2021). YAP1 is expressed intensely in numerous types oftumors, where it has a prime role in tumorigenesis, propagation, and invasion by enhancing cell proliferation (Collak et al., 2020). In the current study, YAP1 showed high immunohistochemical reactivity in 57.3% of UC. Similarly, Liu et al. demonstrated that YAP1 is strongly expressed in 53.1% of UC (Liu et al., 2013). Moreover, Xu et al. stated that YAP1 has elevated expression in bladder carcinoma, and blocking YAP1 significantly inhibits cell proliferation and enhances apoptosis (Xu et al., 2020).

Regarding YAP1 expression relation with tumor size, it was found that high YAP1 reactivity was significantly found with large-sized tumors in UC. Similarly, Ibrahim et al. found that YAP1 expression was significantly related to large tumor size in UC (Ibrahim et al., 2022). On the contrary, Liu et al. found that YAP1 expression didn't differ significantly among different tumor sizes in UC (Liu et al., 2013).

In the current study, it was found that YAP1 expression is significantly related to the tumor grade in which high-grade UC showed a higher frequency of YAP1 expression than low-grade tumors. Supporting our findings, several previous studies reported a YAP1 overexpression in poorer degrees of tumor differentiation in UC (Liu et al., 2013, Li et al., 2015 and Ibrahim et al., 2022). When the Hippo pathway is dysfunctional, YAP1 is abnormally stimulated, which might lead to tumorigenesis, tumor cell stemness, and dedifferentiation

(Shen et al., 2023). This fact supports our finding of increased YAP1 expression in high-grade UC compared to low-grade ones.

Muscle-invasive tumors displayed high YAP1 expression in most tumors compared to a minor percentage with high YAP1 expression among non-muscle invasive tumors. Similar results were reported by Liu et al., who observed that overexpression of YAP1 was significantly correlated with a higher T stage (Liu et al., 2013). Also, Ibrahim et al. found that YAP1 expression had a significant association with advanced tumor stage (Ibrahim et al., 2022).

In the present study, higher YAP1 expression was associated with advanced N stage in which all N1 and N2 UC significantly showed high YAP1 expression. Parallel to our results, Liu et al. reported that high YAP1 expression was significantly correlated with a higher N stage in UC (Liu et al., 2013). Similar results were obtained by previous studies on several solid malignant tumors such as papillary thyroid (Cha et al., 2021), and breast carcinomas (Abdelhafez et al., 2023), in which high YAP1 expression was associated with aggressive tumor features and the presence of nodal metastasis.

With the development of tumor immunotherapy, the function of tumor-associated macrophages (TAMs) in malignant cancers has eventually become a hot spot for its targetability (Chen et al., 2019). CD68 is a pan macrophage marker and the correlation between CD68 expression, and the prognosis of malignant tumors appears to be variable.

Regarding the relation between $CD68^+$ TAMs mean count and tumor size; it was found that high $CD68^+$ TAMs mean count was significantly accompanied by larger tumor size in UC. This can be explained by the role of TAMs in the progression and growth of carcinomas as they are recruited to sites of hypoxia stimulating the transcription factor HIF-1, which activates the expression of the proangiogenic growth factors that aids in tumor cell proliferation through angiogenesis (He et al., 2021)

All noninvasive papillary UC showed low CD68+ TAMs mean count, while most invasive UC showed high CD68+ TAMs mean count. Likewise, Fu et al. reported that TAMs are one of the most abundant infiltrating immune cells of UC with a high density in the tumor core of MIBC (Fu et al., 2018).

A significant statistical difference between conventional UC on the one hand, and UC with squamous differentiation and plasmacytoid type, on the other hand, was elicited. However, no significant statistical difference in CD68+ means count between conventional UC and other subtypes was found. This variability in the count of CD68+ TAMs between UC subtypes may be due to the molecular differences between different histological types of UC with subsequent different immunological responses (Sjödahl et al., 2014).

Despite that no low-grade UC displayed a high CD68+ TAMs mean count and most high-grade UC showed a high mean count, no significant statistical relation was found between CD68+ TAMs mean count and the tumor grade in the present work. Nearly like our finding, previous studies reported that high-grade UC showed higher CD68 expression compared to low-grade ones but with a significant statistical relationship (Boström et al., 2015, Harras and Abo Safia, 2021). Whereas other studies reported that high infiltration of the UC tissue by CD68+ TAMs showed no significant correlation with tumor grade (Ajili et al., 2013 and Sjödahl et al., 2014). This discrepancy may be attributed to the difference in assessing the CD68+ TAMs regarding the methodology adopted in counting or the location studied, peritumoral or intra-tumoral.

In the present work, a significant statistical relation between $CD68^+$ TAMs mean count and pT stage was detected, in which muscle-invasive UC displayed high CD68+ TAMs mean count in most tumors compared to only a minor percentage of non-muscle invasive tumors showed high CD68+ TAMs mean count among. This could be attributed to the fact that TAMs play a crucial role in tumor cell invasion (Mantovani and Sica, 2010). Besides, TAMs can release proteases that degrade the extracellular matrix and thus enhance the tumor cell invasion (Jackute et al., 2018).

On studying the correlation between YAP1 and CD68+ TAMs mean count, a statistically significant positive correlation between both

biomarkers was found. The impact of YAP1 on the recruitment of TAMs depends mainly on controlling the levels of CSF-1 and IL-6 released by the tumor cells causing tumorigenesis and remodeling the composition of TME (Yang et al., 2020).

Interestingly, malignant cells can stimulate YAP1 expression in macrophages to foster their microenvironment. This was proved in triple-negative human breast cancer in which malignant cells activate YAP1 in co-cultured macrophages, which in turn initiated their polarization and promoted metastasis cancer cells (Baroja et al., 2024). The pathogenesis of malignant tumors is sophisticated, and it is ineffectual to inhibit only one signaling pathway to treat tumors (Zhang et al., 2022). Thus, a combination of anti-YAP1 therapeutic agents with other potential targets such as TAMs could act synergistically against malignant urothelial cells.

In short, YAP and TAMs are intimately related and are linked to tumor proliferation, infiltration, and metastasis. A deeper insight into the effect of YAP1 and TAMs on the tumor microenvironment is crucial for understanding their roles in tumor development, for individual assessment of the prognosis of patients, even those with the same histological type and tumor stage, and for designing antitumor multi-target therapeutic modalities, thus effectively improve prognosis as well as the survival of patients with UC.

In conclusion, High YAP1 expression and $CD68^{+}$ TAMs high mean count was significantly related to adverse pathological factors, large-sized tumors, tumors of high grade, and advanced pT stage. Their expression in UC could be used in identifying patients at increased risk of tumor invasion and progression.

CONFLICTS OF INTEREST

All authors declared no conflicts of interest.

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AUTHORS' CONTRIBUTIONS

All authors contributed to the selection and diagnosis of cases. In addition, they contributed to writing the manuscript, performing histopathological examination, and assessing the immunohistochemical results.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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