Expression of aquaporin 3 and vascular endothelial growth factor in transitional cell carcinoma-urinary bladder: an immunohistochemical study

Heba M. Rashad and Sarah N. Nasif

Introduction **Dysregulation of aquaporins (AQPs) is thought to be involved in urothelial carcinogenesis and progression. Angiogenesis is necessary for the growth and subsequent metastasis of transitional cell carcinoma. However, whether there is a positive association in transitional cell carcinoma has not yet been established. This research was conducted to analyze the expression of AQP3 and vascular endothelial growth factor (VEGF) in transitional cell carcinoma with a view toward its development and progression and to correlate them with clinicopathological parameters.**

Patients and methods **This is a retrospective, selected, controlled study carried on 55 pure transitional cell carcinoma cases from January 2010 to December 2017 to assess the immunohistochemical expression of the AQP3 and VEGF and to correlate them with various clinicopathological parameters.**

Results **AQP3 expression showed positive expression in normal urothelium (100%), 90% in carcinoma *in situ* (CIS) cases, whereas in transitional cell carcinoma, it was detected in 52.7% of cases. AQP3 expression is lower in transitional cell carcinoma cases than CIS and normal mucosa ($P < 0.05$). VEGF was not detected in either normal urothelium or CIS, whereas 39 cases of transitional cell carcinoma (70.1%) showed positive expression. Expression of VEGF was significantly higher in bladder cancer specimens than that of normal urothelium and CIS ($P < 0.01$). AQP3 expression was inversely correlated with grade of urothelial cancer ($P = 0.711$), tumor size ($P = 0.272$), depth of invasion ($P = 448$) and TNM stage ($P = 0.364$). VEGF was significantly correlated with the grade of transitional cell carcinoma ($P = 0.332$), depth of invasion ($P = 0.290$), and TNM stage ($P = 0.456$). A significant correlation was detected between AQP3 and VEGF expression in transitional cell carcinoma.**

Conclusion **The results suggested that AQP3 expression to be involved in transitional cell carcinogenesis and progression via its association with VEGF. *Egypt J Pathol* 00:000–000 © 2019 Egyptian Journal of Pathology.**

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Keywords: aquaporin 3, transitional cell carcinoma, vascular endothelial growth factor

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Introduction

Cancer of the urinary bladder represents the ninth most common cancer worldwide and the 14th most common cause of cancer-related death. Transitional cell carcinoma ranks top as the most common malignant tumor in urinary system (Mahdavifar *et al.*, 2016; Siegel *et al.*, 2016).

Transitional cell carcinoma is polygenetic in characteristic and is more likely to recur after the local resection of visible tumors. Early diagnosis and immediate discovery of recurrence could prolong patients' survival time, which was of great significance in the treatment of bladder transitional cell carcinoma (Taylor and Kuchel, 2009; Liu *et al.*, 2015).

Aquaporins (AQPs) are a family of 13 small hydrophobic, channel-forming membrane proteins having the role of transepithelial fluid transport occurring in the urinary concentrating mechanism and glandular fluid secretion (Huber *et al.*, 2012). Recent studies showed that AQP3 is dysregulated in different tumors such as colon cancer playing key roles in cell proliferation and migration (Kang *et al.*, 2015).

Vascular endothelial growth factor (VEGF) is considered the most important angiogenic stimulators during tumor angiogenesis (Verma *et al.*, 2011). VEGF is expressed in bladder tumors, and the increased expression of VEGF is associated with higher tumor stage and progression (Kopparapu *et al.*, 2013).

Many studies demonstrated that AQPs and VEGF were coexpressed in edematous, brain tissue and glioma, as AQP is a small hydrophobic integral membrane protein that acts as a regulator of water balance for the cell, so is associated with significant peritumoral edema, and VEGF has the ability to induce angiogenesis and increase vascular permeability (Wang *et al.*, 2011). However, whether they are coexpressed in transitional cell carcinoma was not investigated.

This study aimed at assessment of immunohistochemical (IHC) expression of AQP3 and VEGF in transitional cell carcinoma and correlated them with clinicopathological parameters.

Patients and methods

This is a retrospective, selected, controlled study carried out on 55 specimens of formalin-fixed, paraffin-embedded transitional cell carcinoma obtained from patients who underwent partial and radical cystectomies. Associated carcinoma *in situ* (CIS) was seen in eight cases adjacent to tumor tissue. Six cases of apparently normal tissue were used as a control group. They were collected from Archives of Pathology Department and Early Cancer Detection Unit, Benha Faculty of Medicine, Benha University, from January 2010 to Decem ber 2017. In each case, clinicopathologic findings, including age, sex, lymph node and distant metastasis status, were obtained from the patients' information system at Pathology Department. All are approved by ethical committee.

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Histopathological study

Paraffin blocks were collected, and three slides of each block of 4-μm thickness were cut, one on plane slide and two on positive charged slides. The sections were dewaxed at 56°C for 2 h, and afterward one slide was made ready for staining with hematoxylin and eosin. Each slide was examined by two specialists for (i) confirmation of the previous diagnosis, (ii) assessment of tumor grade according to the WHO (Moch *et al.*, 2016), (iii) assessment of pathological T stage (depth of invasion), and (iv) assessment of other histopathological features, such as lymph node and distant metastasis. Stage was defined according to American Joint Committee on Cancer on Cancer Criteria (2017).

Immunohistochemical study

For IHC staining, two positive slides were prepared. They were immunostained for AQP3 antibody (Novus Biologicals, Centennial, Colorado, USA), and VEGF antibody (Novus Biologicals). 3,3′-Diaminobenzidine was used as a chromogen. IHC staining was performed using a detection kit (Thermoscientific, Fremont, California, USA) according to the manufacturer's data. A negative control was used for each marker, by omitting the primary antibody and replacing it with normal rabbit serum immunoglobulin G. The details of antibodies are shown in Table 1.

Immunohistochemical assessment

Analysis of aquaporin 3 immunostaining

In cases of complete lack of immunoreactivity, expression was considered AQP3-negative. In cases with partial membranous expression, expression was considered AQP3-positive (Rubenwolf *et al.*, 2012).

Analysis of vascular endothelial growth factor immunostaining

VEGF expression was detected in the cytoplasm of the cells. The percentage of positive cells was calculated as follows: 0, <10%; 1, 10–25%; 2, 25–50%; and 3, >50% of the tumor cells were positive. The staining intensity was scored as: 0, negative immunostaining; 1, weak intensity; 2, moderate intensity; and 3, strong intensity. The sum of the two parameters varied between 0 and 6. The scores from 0 to 2 were considered negative, and scores from 3 to 6 were considered positive (Zhao *et al.*, 2012).

Statistical analysis

Results were analyzed using SPSS (version 16.0.1; SPSS Inc., Chicago, Illinois, USA) statistical package for Microsoft Windows. The Pearson correlation coefficient was used for statistical analysis. *P* value less than 0.05 was considered statistically significant and highly statistically significant when it was less than 0.01.

Table 1 Study markers

Antibody	Type	State	Positive control	Incubation (h)
AQP3	Rabbit polyclonal	Concentrated (1 : 200)	Renal tumor	1
VEGF	Mouse monoclonal	Concentrated (1 : 200)	Hemangioma	1

AQP3, aquaporin 3; VEGF, vascular endothelial growth factor.

Results

Clinicopathological data

The age of the studied cases ranged from 35 to 60 years (mean = 48.3). Male-to-female ratio was 2.2 : 1. All clinicopathological data were summarized in Table 2.

Immunohistochemical results

Analysis of aquaporin 3 and vascular endothelial growth factor immunostaining in studied transitional cell carcinoma cases

AQP3 expression showed positive complete membranous expression in normal urothelium (100%), 75% in CIS cases, whereas in transitional cell carcinoma cases, partial membranous expression was detected in 52.7% of cases. AQP3 expression is lower in transitional cell carcinoma cases than CIS and normal mucosa ($P < 0.05$) (Figs 1 and 2).

Analysis of vascular endothelial growth factor immunostaining in studied transitional cell carcinoma cases

Normal urothelium and CIS cases were negative for VEGF, whereas 39 (70.1%) transitional cell carcinoma cases showed positive expression (Fig. 3). Expression of VEGF was significantly higher in bladder cancer specimens than that of normal urothelium and CIS ($P < 0.01$).

Correlation between aquaporin 3 and vascular endothelial growth factor to clinicopathological parameters

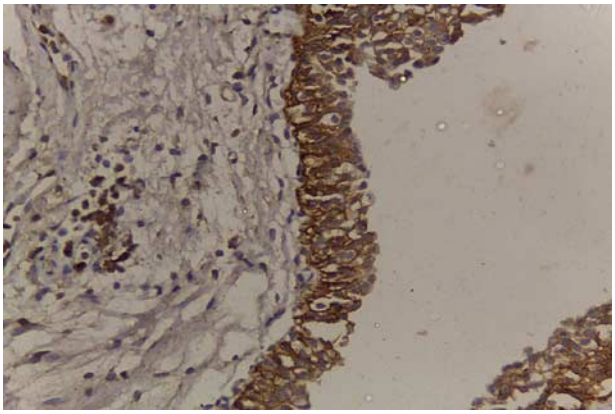
AQP3 expression was inversely correlated with grade of transitional cell carcinoma ($P = 0.711$), tumor size ($P = 0.272$), depth of invasion ($P = 448$), and TNM stage ($P = 0.364$). However, no significant correlation was detected between AQP3 and other variables such as age, sex, lymph node

Table 2 Clinicopathological variables of studied urothelial carcinoma

Clinicopathological data	*N* = 55 (100%) [*n* (%)]
Age (years)	
≤50	12 (21.8)
>50	43 (78.2)
Sex	
Male	38 (69.1)
Female	17 (30.9)
Grade	
2	27 (49.1)
3	28 (50.9)
Size (cm)	
<3	18 (32.7)
>3	37 (67.3)
T	
1	2 (3.6)
2	12 (21.8)
3	37 (67.3)
4	4 (7.3)
LN	
N0	29 (52.7)
N1	26 (47.3)
DM	
M0	39 (70.9)
M1	16 (29.1)
TNM stage	
I	2 (3.6)
II	16 (29.1)
III	22 (40)
IV	15 (27.3)
Bilharziasis	
Absent	42 (76.4)
Present	13 (32.6)

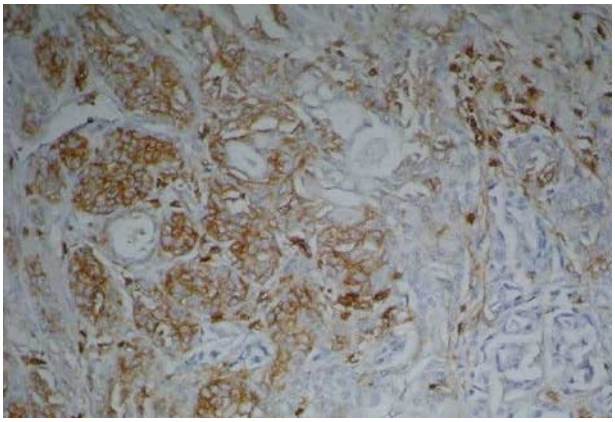
DM, distant metastasis; LN, lymph node; T, depth of invasion.

Fig. 1



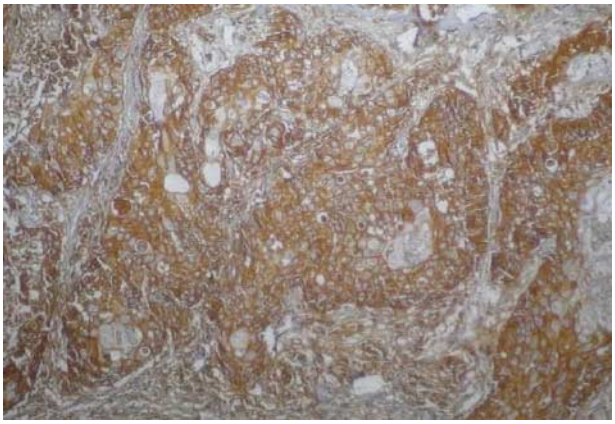
Normal urothelium, showing positive membranous aquaporin 3 expression (immunohistochemistry, ×40).

Fig. 2



Transitional cell carcinoma- positive for aquaporin 3 showing partial aquaporin 3 expression (immunohistochemistry, ×10).

Fig. 3



Transitional cell carcinoma positive for vascular endothelial growth factor (immunohistochemistry, ×40).

metastasis, distant metastasis, or associated bilharziasis (Table 3).

VEGF expression was significantly correlated with grade of transitional cell carcinoma ($P = 0.\ 332$), depth of invasion ($P = 0.290$), and TNM stage ($P = 0.\ 456$). However, no significant correlation was detected between VEGF and other variables such as age, sex, tumor size, lymph node metastasis, distant metastasis, or associated bilharziasis (Table 3).

Correlation between Aquaporin 3 and Vascular endothelial growth factor in studied transitional cell carcinoma cases

Aquaporine 3 expression was significantly correlated with VEGF in transitional cell carcinoma cases ($P = 0.001$) (Table 4).

Discussion

AQP3 is a member of channel-forming membrane proteins functioning as transepithelial fluid transport (Verkman, 2012). The expression of AQP3 in tumor cells of different tissue origins was evaluated in many studies (Ishimoto *et al.*, 2012).

In this study, AQP3 expression is lost in transitional cell carcinoma, whereas it was retained in CIS and normal urothelium, indicating that the lack of expression contributes to the progression of transitional cell carcinoma. These results were in line with Otto *et al.* (2012) and Breyer *et al.* (2017). In contrast, Ji *et al.* (2008) stated that AQP3 is overexpressed in ovarian carcinoma and facilitated the migration of cancer cell. Moreover, in squamous cell carcinoma of skin, Hara-Chikuma and Verkman (2008) reported AQP3 overexpression. This conflict was explained by Rubenwolf *et al.* (2014) by the hypothesis that there is a unique pattern of AQP3 expression during normal human urothelial cytodifferentiation, which is limited to the differentiated phenotype of the cells, and the loss of differentiation may be paralleled by the loss of AQP3 expression in tumor cells. Hence, dysregulation during urothelial carcinogenesis is different from the changes seen in other tumor tissues.

In this study, AQP3 expression was significantly correlated with the grade of transitional cell carcinoma ($P = 0.711$). This result matches the result of Rubenwolf *et al.* (2014). One of the possibility of such a correlation may be that loss of AQP3 creates tumorigenic effect through activation of matrix metalloproteinase (Xu *et al.*, 2011). In contrast, Breyer *et al.* (2017) concluded that the loss of AQP3 expression associated with tumor cell invasion into the submucosa and mucosa irrespective of tumor differentiation, and this difference may be owing to that they depended on their results on only 14 specimens of invasive transitional cell carcinoma and associated CIS, and they found that all CISs have intense AQP3 expression; this was surprising to them as CIS is commonly regarded as a precursor of the poorly differentiated non-papillary carcinoma phenotype. In our opinion and supported by the hypothesis that in human urothelium, expression and function of AQP3 are associated with the differentiated phenotype, there may be a role of AQP3 loss and progression from CIS to transitional cell carcinoma in both invasion and differentiation.

Table 3 Correlation between aquaporin 3 and vascular endothelial growth factor with clinicopathological parameters

	Aquaporin 3 [*n* (%)]			VEGF [*n* (%)]		
	Negative	Positive	*P* value	Negative	Positive	*P* value
Age (years)						
≤50 (*n*=12)	5 (41.7)	7 (58.3)	0.667	4 (33.3)	8 (66.7)	0.721
>50 (*n*=43)	21 (48.8)	22 (51.2)		12 (27.9)	31 (72.1)	
Sex						
Male (*n*=38)	18 (47.1)	20 (52.6%)	0.983	10 (26.3)	2 (73.7)	0.507
Female (*n*=17)	8 (47.1)	9 (52.9)		6 (35.3)	11 (64.7)	
Tumor size (cm)						
≤3 (*n*=18)	5 (27.8)	13 (72.2)	0.272*	8 (44.4)	10 (55.6)	0.083
>3 (*n*=37)	21 (56.8)	16 (43.2)		8 (21.6)	29 (78.4)	
Grade						
Grade 2 (*n*=27)	3 (11.1)	24 (88.9)	0.711**	12 (44.4)	15 (55.6)	0.332*
Grade 3 (*n*=28)	23 (82.1)	5 (17.9)		4 (14.3)	24 (85.7)	
T						
1	0	2	0.448**	2	0	0.290*
2	2 (16.7)	10 (83.3)		4 (33.3)	8 (66.7)	
3	20 (54.1)	17 (45.9)		10 (27)	27 (73)	
4	4	0		0	4	
LN						
N0 (*n*=25)	13 (52)	12 (48)	0.530	9 (36)	16 (64)	0.312
N2 (*n*=30)	13 (43.3)	17 (56.7)		7 (23.3)	23 (76.7)	
DM						
M0 (*n*=39)	17 (43.6)	22 (56.4)	0.402	13 (33.3)	26 (66.7)	0.288
M1 (*n*=16)	9 (56.2)	7 (43.8)		3 (18.8)	13 (81.2)	
TNM stage						
I (*n*=2)	0	2 (100%)	0.364**	2 (100)	0	0.456**
II (*n*=16)	4 (25)	12 (75)		8 (50)	8 (50)	
III (*n*=22)	12 (54.5)	10 (45.5)		5 (22.7)	17 (77.3)	
IV (*n*=15)	10 (66.7)	5 (33.3)		1 (6.7)	14 (93.3)	
Bilharziasis						
Present (*n*=13)	5 (38.5)	8 (61.5)	0.476	3 (23.1)	10 (76.9)	0.593
Absent (*n*=42)	21 (50)	21 (50)		13 (31)	29 (69)	

DM, distant metastasis; LN, lymph node; T, depth of invasion; TNM, depth of invasion, lymph node, distant metastasis; VEGF, vascular endothelial growth factor.
*Correlation is significant at the 0.05 level (two tailed).
**Correlation is significant at the 0.01 level (two tailed).

Table 4 Correlation between aquaporin 3 and vascular endothelial growth factor in studied transitional cell carcinoma cases

	VEGF [*n* (%)]	
Aquaporin 3	Negative	Positive
Negative		
26	2 (7.7)	24 (92.3)
Positive		
29	14 (48.3)	15 (51.7)
P value	0.001**	

VEGF, vascular endothelial growth factor.
**Correlation is significant at the 0.01 level (two tailed).

This study demonstrated that AQP3 expression was significantly associated with TNM stage ($P=0.364$). Similar results were obtained by Rubenwolf *et al.* (2014) and Breyer *et al.* (2017), indicating the role of AQP3 loss with progression toward muscle-invasive disease and worse outcomes.

VEGF, is considered the most potent angiogenetic factor known responsible for angiogenesis (Fauconnet *et al.*, 2009). In this study, VEGF expression was significantly higher in bladder cancer specimens than that of normal mucosa and CIS ($P<0.01$).

Similar results were obtained by Yang *et al.* (2004) and Al-Abbasi *et al.* (2009) who revealed significant differences between normal urothelium and cancer tissue for VEGF expression, indicating that angiogenesis participates in tumor development and progression.

In this work, VEGF expression significantly correlated with the grade of transitional cell carcinoma. This was compatible with Rahmani *et al.* (2012).

According to our results, VEGF was associated with increasing tumor stage. Similar findings were obtained by Tian *et al.* (2015), indicating the role of VEGF in the progression of transitional cell carcinoma and its importance as a prognostic indicator.

In this study, an association of AQP3 and VEGF was detected, suggesting that AQPs might cross-talk with VEGF, facilitating angiogenesis. This could be explained through that AQPs may interact with the VEGF signaling pathway to regulate fluid transport and lymphangiogenesis, contributing in the occurrence and progression of transitional cell carcinoma. In conclusion, this study demonstrated that lack of AQP expression may be involved in carcinogenesis and progression of transitional cell carcinoma through interaction with VEG; thus, AQP3 may be considered a novel therapeutic target for transitional cell carcinoma.

Conflicts of interest

There are no conflicts of interest.

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