ORIGINAL ARTICLE

Evaluation of vascular endothelial growth factors A, C and D as indicators of lymphangiogenesis and angiogenesis in invasive and non-invasive urothelial carcinoma bladder

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Abstract

Objective: To study the immunohistochemical expression of vascular endothelial growth factors in urothelial tumours of bladder and its possible association with tumour characteristics and microvessel density.

Methods: The cross-sectional descriptive study was conducted at the Histopathology Department of the Armed Forces Institute of Pathology, Rawalpindi, Pakistan, from July 2011 to December 2012, and comprised cases of non-invasive and invasive urothelial tumours of the bladder. The microvessel density and expression of vascular endothelial growth factors A, C, D were evaluated by immunohistochemistry. Specimens of transurethral bladder biopsies and surgical resection were examined. The cases were classified into non-invasive (stage pTa) and invasive groups as well as low-grade and high-grade groups. The presence of in-situ component was evaluated in each category. To assess the microvessel density, highly vascularised foci ('hot spots') after immuno-staining with CD34 were quantified for number of vessels per square millimetre and for vascular surface area density. No distinction was made between lymphatic and blood vessels. Vascular endothelial growth factor staining was scored semi-quantitatively.

Results: The study examined 100 histopathology specimens, including 90(90%) transurethral bladder biopsies and 10(10%) surgical resection specimens of bladder. There were 45(45%) non-invasive (stage pTa) cases and 55(55%) invasive (stage pT1-4) cases. Besides, there were 43(43%) low-grade (grades 1 and 2) cases, and 57(57%) high-grade (grade 3) cases. Vascular endothelial growth factors A, C and D staining scores showed positive association with stage ($p=0.02$;$p<0.01$; $p<0.01$)and grade ($p=0.007$;$p=0.004$; $p=0.002$) of the tumour. Tumours with in-situ component showed association with number of vessels per square millimetre ($p<0.01$) and vascular surface area density ($p=0.02$).

Conclusions: Parameters like vascular endothelial growth factor and microvessel density need to be studied further for selection of cases with potential for targeted therapy.

Keywords: Urothelial carcinoma, Papillary urothelial neoplasm, Microvessel density, Lymphangiogenesis, Angiogenesis, Vascular endothelial growth factors. (JPMA 65: 851; 2015)

Introduction

The histological classification of urinary bladder tumours is simple and majority of bladder tumours are of epithelial origin. In 2008 about 386,000 new cases of bladder cancer were reported worldwide and approximately 150,000 deaths resulted from urothelial tumour of bladder. According to World Cancer Report 2003 and World Health Report 2004, urothelial carcinoma was among the most frequent causes of death. According to reports published by the tumour registry at the Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan, tumours of urinary bladder have been listed among top 10 tumours.[1,2] In addition to tumour stage, histological grade, extent of invasion, lymph node metastasis, Vascular endothelial growth factors (VEGFs) are also being studied as independent prognostic and therapeutic markers. Tumour angiogenesis, as assessed by microvessel density (MVD), shows significant correlation with poor prognosis in several human cancers, including urological cancers. Angiogenesis is a necessary process for growth, development and repair. The vasculature is kept in a stable state by the intricate balance between pro-angiogenic and anti-angiogenic factors.[3] The process of tumour vascularisation can be divided into initial prevascular phase known as angiogenic switch, leading to the phase of neovascularisation. The mechanism of tumour lymphangiogenesis and angiogenesis is complex. It has been observed that VEGFs play a apivotal role in tumour neovascularisation and tumour progression. The vascular growth factor family includes VEGF-A, C, D and placental growth factor. These factors bind their respective receptors located on the endothelial cells

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(VGEF receptor,VEGFR-1, VEGFR-2 and VEGFR-3).[4] The VEGFs expression and MVD is increased in various tumours. The assessment of MVD has shown strong correlation with worse prognosis in tumours like breast carcinoma, carcinoma of prostate, renal cell carcinoma and ovarian cancers.[5,6] VEGFs are being tried as proangiogenic factor to treat ischaemic processes and anti-VEGFs have proved a role in the treatment of certain cancers. Although VEGF-C and VEGF-D are considered primary lymphangiogenic factors, but they also play some role in angiogenesis like VEGF-A. Each member of VEGF family play some role both in lymphangiogenesis and angiogenesis.[7] The inhibition of VEGF-C and VEGF-D receptor, signal transduction may prevent the possible development of metastasis in cancer.[8] The assessment of microvasculature can be done by immunohistochemical (IHC) staining of selected formalin-fixed paraffin-embedded tumour sections. CD34 and anti-VEGF antibodies can be used to study MVD and VEGF expression respectively. The images can be analysed for quantitative estimation of MVD in terms of vascular surface area density (VSD)and number of vessels per square millimetre (NVS) in selected tumour foci.[8] There are conflicting reports about the correlation of MVD and tumour prognosis. The observed discrepancies are probably due to different methods used by investigators for the quantitative assessments.

The current study was planned to observe the IHC expression of VEGFs in urothelial tumours of bladder and its possible association with tumour characteristics and MVD.

Material and Methods

The cross-sectional descriptive study was conducted at the Histopathology Department of AFIP, Rawalpindi, Pakistan, from July 2011 to December 2012, and comprised cases of non-invasive and invasive urothelial tumours of the bladder. The MVD and expression of VEGF-A, C, D were evaluated by IHC. Specimens of transurethral bladder biopsies and surgical resection were examined.

Patients with a history of any other malignancy, including a prior history of urinary tract malignancies and patients who received neoadjuvant therapy were excluded. The selected cases were evaluated by two pathologists using tumour grading and staging according to the World Health Organisation (WHO) and International Society for Urological Pathology (ISUP) classification system.[9] The cases were classified into non-invasive urothelial carcinoma (stage pTa) and invasive urothelial carcinoma (pT1-4) groups as well as low-grade (grades 1 and 2) and high-grade (grade 3) groups.

After receiving approval from the AFIP ethical committee, all biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin using standard tissue processing protocols. Diagnoses were established after examining Haematoxylin-Eosin (H&E)-stained slides. Olympus DPX12 camera was used for digital micrographs and image J was used for analysis of selected foci in tumour and in adjacent urothelium showing in-situ changes. VEGF-A, VEGF-C and VEGF-D staining expression in tumour cells was studied by using polyclonal antibodies (Gene Tex, Inc). CD34 expression was studied in endothelial cells by using monoclonal anti bodies (Vision Biosystems Novocastra). On routine examination sections were selected for various grades of urothelial carcinoma, presence or absence of lamina propria and muscle invasion, vascular, lymphatic and neural invasion. Foci with maximum vascular density, including blood vessels and lymphatics, were selected as hot spots. IHC was performed on a single representative block from each case. Direct microscopy and Image analysis was done on sections immuno-stained with CD34, VEGF-A, C and D antibodies. The tumour 'hot spot' were chosen at low magnification and vessels stained with CD34 endothelial cells were counted in a representative high magnification. In addition to stained micro vessels, single endothelial cells or small clusters immuno-stained with CD34 were considered individual microvessel. The large vessels were disregarded. As CD34 cannot differentiate between lymphatic and blood vessel tumour, MVD was analysed without discrimination between lymph vessels and micro-blood vessels. The average number of microvessel per high magnification after examining three representative foci was calculated. For quantification of NVS, Image J was used on selected photomicrographs. The area occupied by CD34-positive microvessel and total tissue area per section were quantified for VSD. The staining expression of VEGF-A, VEGF-C, and VEGF-D was assessed semi-quantitatively. The percentage of positive cells was calculated (approximately 500 neoplastic cells were examined in each case). Tumour foci were considered positive when more than 50% of cells were clearly stained.[10] The staining intensity was divided into four grades, none, weak, moderate, and strong, scoring 0 to 3 respectively.[11,12]

Sample size determination was based on non-invasive tumour stage in low-grade urothelial tumours reported as 36.4%[13] with margin of error 9.5% and 95% confidence interval (CI). A total sample size of 100 patients was calculated.

Data was analysed using SPSS 21. Descriptive statistics was used to summarise the continuous variables like age,

NVS and VSD. And they were reported as mean± standard deviation (SD). While categorical variables like tumour characteristics, histological grade, classification, muscular, vascular, lymph node, and perineural invasion, were presented as frequencies and percentages. To compare the mean difference, independent" test and analysis of variance (ANOVA) were used for continuous variables. For categorical variables Fischer's Exact test and Chi-Square independent test were used to compare the proportion difference between variables. Multiple logistic regression test was applied to determine possible association between VEGF-A, C, D staining scores and MVD. For statistical analysis the staining expression was divided in two groups. Cases with s0 and 1 (none and weak IHC staining) were considered insignificant expression and cases with score 2 and 3 (moderate, and strong IHC staining) were considered with significant expression. P<0.05 was considered significant.

Results

The study examined 100 histopathology specimens, including 90(90%) transurethral bladder biopsies and 10(10%) surgical resection specimens of bladder. Overall mean age of the patients was 51.08±10.84 years (rang: 30-79 years) (Table-1). There were 45(45%) non-invasive (stage pTa) cases and 55(55%) invasive (stage pT1-4) cases. Besides, there were 43(43%) low-grade (grades 1 and 2) cases, and 57(57%) high-grade (grade 3) cases.

The foci of micro-vessels, including blood vessels and lymphatic vessel, were highlighted in CD34 IHC staining.

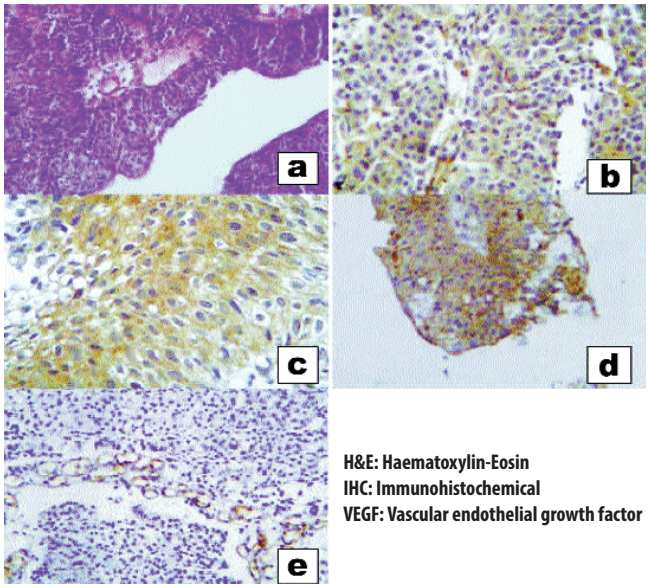

Figure: Low grade papillary urothelial neoplasm. a) H&E stain (x20). b) VEGF-A IHC expression (x40). c) VEGF-C IHC expression (x40). d) VEGF-D IHC expression (x10). e) CD34 IHC expression (x20).

Table-1: Clinico-pathological characteristics (n=100).

Variables	Mean (±S.D)	N	(%)
Age (years)	51.08 (± 10.48)	-	-
Gender			
Male	-	76	(76.00)
Female	-	24	(24.00)
Specimens			
TUR	-	90	(90.00)
Others	-	10	(10.00)
Tumour grade/stage			
Invasive	-	55	(55.00)
Noninvasive		45	(45.00)
High grade	-	57	(57.00)
Invasive	-	49	(86.00)
Noninvasive	-	8	(14.00)
Low grade	-	43	(43.00)
Invasive	-	6	(14.00)
Noninvasive		37	(86.00)
VSD	148.22 (±8.35)	-	-
NVS	59.38 (±5.19)	-	-
VEGF-A			
Score 1	-	31	(31.00)
Score 2	-	39	(39.00)
Score 3	-	30	(30.00)
VEGF-C			
Score 1	-	3	(3.00)
Score 2	-	63	(63.00)
Score 3	-	34	(34.00)
VEGF-D			
Score 1	-	3	(3.00)
Score 2	-	70	(70.00)
Score 3	-	27	(27.00)
In-situ			
Yes	-	18	(18.00)
No	-	82	(82.00)

TUR: 9 SD: Standard deviation
NVS: Number of vessels per square millimetre of tumour
VSD: Vascular surface area density VEGF: Vascular endothelial growth factor.

As no specific marker like D2-40 antibody for lymphatics was used, vessels stained with CD34 and without any red blood cell (RBC) in their lumen were considered to be lymphatic and assessed along with other vessels in hot spots. VEGF-A, C and D showed cytoplasmic staining in tumour cells only. The normal urothelium did not show any staining. All tumour sections stained positively, and staining intensity varied between very weak and strong. The majority of high-grade urothelial tumours stained heterogeneously and showed variable intensity within same section and tumour cell foci compared to low-grade tumours (Figures-1 and 2). Among the non-invasive cases, low-grade papillary urothelial neoplasm were 37(82.3%) and high-grade urothelial carcinoma 8(17.7%). Among the invasive cases low-grade were

Table-2: Comparison of vascular parameters NVS, VSD with grade and stage of tumour (n=100).

Tumour stage /grade	NVS Mean (±S.D)	Mean difference	p-value	VSD Mean (±S.D)	Mean difference	p-value
Noninvasive	140.67 (±4.43)	-13.73	<0.01	53.5 (±2.47)	-10.68	<0.01
Invasive	154.40 (±5.05)			64.18 (±2.82)		
High	63.14 (±4.42)	8.74	<0.01	153.35 (±5.96)	11.93	<0.01
Low	54.40 (±3.61)			141.42 (±5.83)		
In-situ						
Yes	156.94 (±5.45)	10.38	<0.01	62.50 (±3.01)	3.71	0.02
No	146.56 (±7.77)			58.79 (±8.20)		

NVS: Number of vessels per square millimetre of tumour VSD: Vascular surface area density
VEGF: Vascular endothelial growth factor P value according to independent sample t 2- Tail test
SD: Standard deviation.

Table-3: Relationship between VEGF staining expression and vascular parameters VSD and NVS (n=100).

Variables	B	p-value	OR	95% C.I
VEGF-A				
VSD	-0.033	0.541	0.96	0.86 - 1.07
NVS	0.171	0.032	1.18	1.01 - 1.38
VEGF-C				
VSD	0.197	0.002	1.217	1.07 - 1.38
NVS	-0.090	0.282	0.914	0.77 - 1.07
VEGF-D				
VSD	0.228	0.001	1.25	1.09 - 1.44
NVS	-0.129	0.159	0.87	0.73 - 1.07

VEGF: Vascular endothelial growth factor. Multiple logistic regression.
NVS: Number of vessels per square millimetre of tumour
VSD: Vascular surface area density
CI: Confidence Interval

6(11%) and high-grade 49(89%). Invasive group included tumours with sub-mucosal invasion (pT1) and tumours with invasion of muscularispropria (pT2 and3).Metastasis in a single lymph node of less than 5cm in size (stage pN2) was seen in 5(9%) cases. The presence of vascular and lymphatic invasion was seen in 29(53%) cases and out of these, 23(80%) were high-grade invasive urothelial carcinoma.

In-situ component was seen in 18(18%) cases (3[16.6%] low grade and 15[83.3%] high grade tumours). Presence of In-situ component did not show significant correlation with tumour grade (p>0.05). Grade of the tumour and presence of In-situ component was significantly associated with NVS and VSD values (p<0.05 each). Significant correlation was observed between presence of tumour invasion and tumour grade (p<0.01). VEGF-A,C and D staining expression scores showed significant correlation with stage of the tumour (p=0.02;p<0.01; and p<0.01 respectively). Similar association was seen between VEGF-A,C and D staining scores and grade of the tumour (p=0.007; p=0.004; and p=0.002 respectively) (Table-2). Association between VEGF-A, C, D staining expression and MVD was also noted (Table-3).

Discussions

Bladder cancer is among the top 10 tumours in Pakistan. Urothelial carcinoma has been reported as the most frequent subtype. For superficial tumours treatment is challenging as there is high risk of recurrence and tumour progression. Bladder preservation with effective transurethral resection (TUR) and intravesical therapy is preferred to radical surgery. Angiogenesis is a constant feature of tumour pathogenesis. It is considered an essential component of tumour progression, making it a possible therapeutic target. Development of new vessels play important role in the development of metastasis.[14-16] Angiogenesis or neoangiogenesis depends upon balance between pro- and anti-angiogenic factors. The study of lymphangiogenesis has been limited due to less knowledge about specific lymphangiogenic growth factor. It is considered that angiogenesis and lymphangiogenesis start together during tumour growth. All the members of VEGF family play a role in this process. To our knowledge there is scant data about the role of VEGFs in urothelial carcinoma. Definite correlation between serum VEGF level and invasiveness of bladder cancer has been observed. It has been observed that in low-grade papillary tumours and carcinoma in situ of urinary bladder there is increased vascularity. This observation indicates that angiogenesis is acquired relatively early during bladder tumour growth.[17] It is considered that VEGF-targeted therapy may be effective in treating invasive bladder cancers. In our study the low-grade papillary urothelial neoplasm as well as high-grade invasive urothelial carcinoma showed increased neovascularisation in terms NVS and VSD. Similar correlation was observed in tumours with in-situ

component. It has been reported that invasive bladder tumours with higher MVD are associated with recurrence and low survival.[18,19] A study observed significant increased microvessel count in invasive tumours of urinary bladder.[20] Another study pointed out difficulty in the assessment of MVD in papillary urothelial carcinoma as it was hard to decide which part of the tumour vasculature to be counted. They studied only solid areas with high-grade tumours. Consequently tumour vascularity was found to be significantly related to survival and grade of tumour.[21] We evaluated the tumour by using hot spot method and observed CD34-stained micro vessels both in papillary and solid areas. In some of the studies, sections were selected to measure intratumoral areas as well as peritumoral areas to quantitate the micro vascular density.[22,23] In our study the expression of VEGF-A, C, D in terms of staining score showed definitive correlation with the stage and histological grade of bladder tumour. The main objective of our study was to evaluate the VEGF-A, C and D expression and its possible association with tumour characteristic and MVD in terms of NVS and VSD in urothelial tumours of bladder.

The concepts of morphometery for the quantitative methods are evolving. Methodology of microvessel assessment as used by various investigators has been blamed for the discrepancies between results from different studies and the controversial data obtained in cases of superficial bladder tumours. Presently there is scant and conflicting data about the role of the VEGF family in urothelial tumours. Most of the studies focused only on VEGF-A as proangiogenic factor. The possible role of VEGF-C and VEGF-D both in angiogenesis and lymph-angiogenesis is being reported. Increased expression of VEGF-C in tumours has been reported to correlate with regional lymph node metastasis. Presently most of the researchers believe that VEGF-A also has some role in the development of lymphatics.[24] Investigators reported that VEGF-C and VEGF-D were positively associated with MVD in cancer tissues without giving any separate account of lymphatics and blood vessels.[25] We studied MVD without discrimination between blood vessels and lymphatics. VEGF-A showed association with NVS whereas VEGF-C and D were associated with VSD. We speculate that VEGFs play a role in tumour neovascularisation. A study on colorectal tumours observed increased VEGF-D expression associated with lymph vessels density only.[16] Another reported correlation of VEGF-C and VEGF-D with lymph vessel density in breast cancer.[26] It is also suggested that any divergent results are possibly because of difference in the various antibodies used, immunostaining techniques and difference in microvascular quantification.[27] Further studies are required to standardise these research tools to get more conclusive results.

Conclusion

VEGFs are expressed by tumour cells both in invasive and non-invasive urothelial tumours. MVD showed significant correlation with grade and stage of urothelial tumours. Significant association of micro-vascular density with in-situ component of urothelial carcinoma possibly correlate with tumour progression both in invasive and non-invasive urothelial carcinoma. VEGF- A expression is associated with NVS whereas VEGF-C and D showed association with VSD. We speculate that possible regulation of VEGFs may provide a useful way of inhibiting the process of tumour lymphangiogenesis and angiogenesis in low-grade as well as high-grade urothelial tumours at early stages.

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