

# IMMUNOHISTOCHEMICAL EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR DOES NOT CORRELATE WITH MICROVESSEL DENSITY IN INVASIVE BLADDER CARCINOMA

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## ABSTRACT

**Objectives:** To investigate the presence or absence of correlation between the immunohistochemical expression of vascular endothelial factor (VEGF) and microvessel density (MVD), and the potential prognostic value patients in bladder carcinoma.

**Methods:** VEGF expression and MVD were assessed on specimens from 50 histological confirmed invasive bladder carcinomas. VEGF expression was estimated as 0 – no positive tumor cells, 1 – less than 10% positive tumor cells, 2 – up to 50% positive tumor cells, and 3 – over 50% positive tumor cells. MVD was calculated on slides stained with CD34 antibody. The arithmetical media were statistically processed with SPSS 17.0, Student test and chi square, and  $p < 0.5$  was considered statistically significant.

**Results:** VEGF was positive in 13.33% of the urothelial carcinomas, and negative in adenocarcinoma and squamous cell carcinoma. In the positive cases the intensity of reaction was weak or moderate in the tumor cells. In the tumor area only the endothelium was positive for CD34, which made it possible to calculate MVD, and to detect vascular invasion. In urothelial invasive carcinoma, the average MVD calculated was 28.6. There was no statistical correlation between MVD, VEGF and tumor prognosis. High values for MVD were correlated with vascular invasion and tumor grading.

**Conclusions:** We found no correlation between VEGF and MVD, or tumor prognosis. MVD is an efficient tool in assessing tumor prognosis. CD34 is a useful marker in detecting vascular invasion, and immature and intermediate blood vessels that could be targets for antivasular therapy.

**Key words:** bladder cancer, immunohistochemistry, microvessel density, prognosis, vascular endothelial growth factor

## INTRODUCTION

Bladder tumors are frequent malignant tumors and from practical point of view they are defined as superficial and invasive. If in superficial tumors therapeutic and follow-up procedures are well standardized and the patients have a favorable prognosis, in patients with invasive urothelial carcinoma the outcome is significantly different. Although invasive tumors are more rare than superficial, the prognosis is significantly worse because they can show lymph node or/and distant metastasis in the moment of diagnosis. Chemotherapy, with or without radiotherapy, have been demonstrated as insufficient to block the tumor growth, and virtually it has no evident effect in cases with metastases.

For more than a century, researches in oncology directly and exclusively focused on malignant cells. In the last three decades there were accumulated a lot of data that support the role of tumor microenvironment not only in the local progression but also in the metastatic process. We investigated the vascular network associated to urothelial carcinoma because blood vessels are key elements in the natural evolution of malignant tumors, and the significance of microvessel density in invasive tumors shows only controversial results.

Angiogenesis is the process by which new blood vessels are formed from preexisting ones, in

both normal and pathological conditions. Somehow surprising, angiogenesis was less investigated in bladder tumors, but preliminary studies have shown that the angiogenic profile of the tumor has predictive impact on local progression, lymph node and distant metastasis. There were published only few articles on MVD in urothelial carcinoma, and even few try to correlation of MVD with VEGF. In the majority of publications high MVD is associated with bad prognosis, correlating with advanced-stage, rapid local progression and metastasis. On the other hand, MVD does not bring information about the potential response to antivasular therapy, and the relation between MVD and angiogenic growth factor is uncertain.

VEGF is a growth factor, being the strongest known angiogenic substance. VEGF is secreted by a large variety of normal cells, but it is also overexpressed by tumor cells, particularly as a consequence of hypoxia generated by rapid proliferation. VEGF promotes proliferation, differentiation, survival and migration of endothelial cells in both normal and pathological conditions. VEGF becomes active after binding specific receptors expressed by endothelial cells, and from these, the most effective is VEGFR2. Although VEGFR2 is intensely expressed by urothelial carcinoma-associated blood vessels, a major antitumor effect of specific inhibitors was not yet demonstrated [1]. In many human

tumors it was found a correlation between angiogenic growth factors and microvessel density, but this is not a general rule [2].

Based on the existing data, the working hypothesis of the present study is to explain the correlation or the lack of correlation between VEGF expression and MVD, results may be useful to identify a therapeutic target in the first condition, or to find out other mechanisms for bladder tumor angiogenesis in the second. This study could be useful to refine personalized therapy and to increase the efficacy of biologic therapies.

## MATERIALS AND METHODS

**Patients.** There were investigated 50 consecutive cases with T2-T4 invasive bladder carcinoma, aged between 54 and 76 years. Diagnosis was based on clinical, imagistic, endoscopic, and pathological procedures. In all patients it was performed radical cystectomy followed by low pressure bladder reservoir. The specimens for the present study were taken from the tumor and also included neighbor apparently normal tissue.

**Primary processing.** Specimens were washed in buffer saline and fixed in buffer formalin for 48 to 72 hours. Paraffin embedding was done using Thermo Shandon system. From each paraffin block there were performed multiple serial sections 3  $\mu$ m thick. Sections stained with hematoxylin-eosin were used for the pathologic diagnosis and evaluation of the grading of differentiation (G).

**Immunohistochemistry** was performed automatically, using Leica Bond-Max system (Leica Biosystems, Newcastle upon Tyne, UK). Paraffin section were submitted to antigen retrieval for 20 minutes (Bond Epitope Retrieval Solution 2, Leica Biosystems, Newcastle Ltd). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes, and then treated with the primary antibody. The working system was the Bond Polymer Refine Detection System, and the final product of reaction was visualized with 3, 3 diamino-benzidine in brown. Nuclei were stained with hematoxylin and finally sections were mounted with Baume of Canada. We used as primary antibodies anti-CD34 (clone QBEnd10, Dako Glostrup, dilution 1:25, antigen retrieval pH6), and anti VEGF-A (clone VG-1, Santa Cruz, dilution 1:25, antigen retrieval pH8).

**Microscopic evaluation and image analysis.** Sections were analyzed with the microscope Zeiss Axiocam 506 (Jena, Germany). MVD was calculated based on the method proposed by Weidner (1991). In brief, two independent observers have choose three microscopic fields at low power magnification with high vascular density on section stained for CD34, for each case. The arithmetical media found at x200 magnification was the value of MVD for the respective case. Both tumor and peritumor areas were taken into account and evaluated.

VEGF reaction was scored as follows: 0 – negative, no tumor cells stained, 1 – less than 10% positive tumor cells, 2 – 11-50%, and 3 – over 50% positive tumor cells. The outer positive control for VEGF immunohistochemical reaction was the normal kidney that shows a strong reaction particularly at tubular level.

**Statistical analysis** was applied to show the relationship between MVD and VEGF expression, using SPSS17.0 soft. Student test and chi square were applied, and  $p < 0.5$  has been considered as statistically significant. A survival analysis was not done, as the follow-up was less than five years in most of the cases

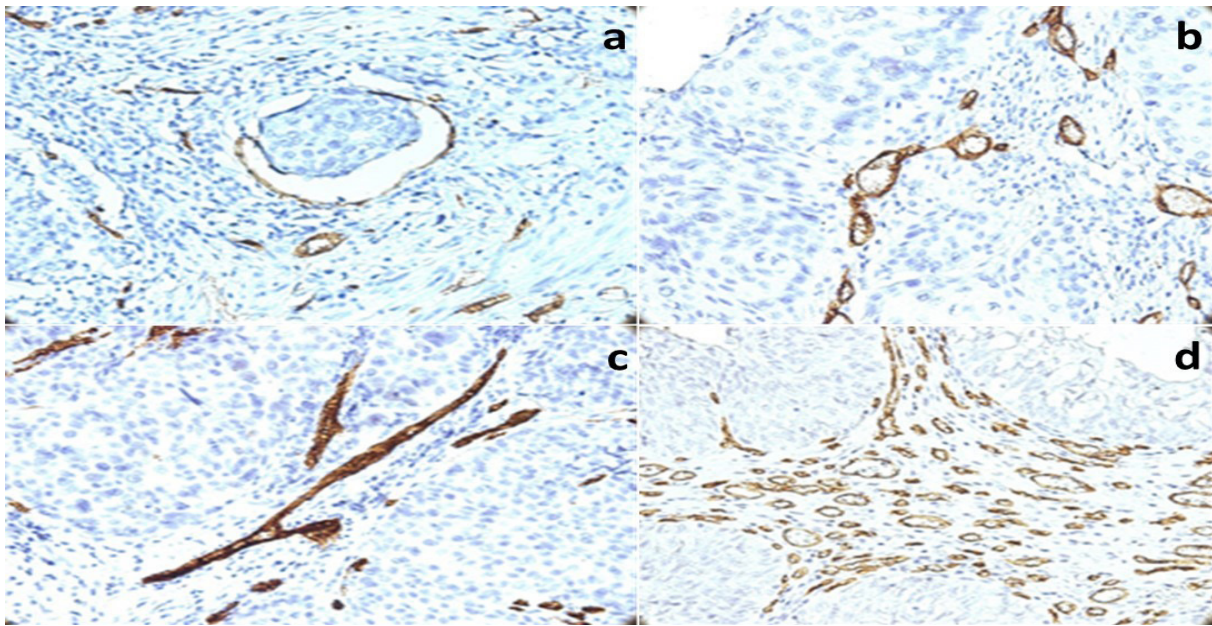
## RESULTS

From 50 cases, we found 44 urothelial carcinoma, 3 adenocarcinoma, and 3 squamous cell carcinoma. In urothelial carcinoma we noticed T2 in 7 cases, T3 in 12, and T4 in 27. We found G1 in 2 cases, G2 in 14, and G3 in 28 cases. All cases with adenocarcinoma and squamous cell carcinoma features were T3 G2 or G3.

VEGF immunohistochemical expression has been investigated in all cases included in the study, and evaluated based on the score mentioned before. Normal and dysplastic urothelium, and papillary proliferation associated to invasive tumors were negative in all cases where they were present. The final product of reaction noticed on the outer control slides was stained in dark brown, with cytoplasmic and granular pattern. From invasive urothelial carcinoma only 5 (13.33%) have shown positive reaction. The maximum achieved score was 3 from 6 possible points. In positive cases the reaction was weak or moderate with heterogeneous distribution in the cytoplasm of tumor cells (**Figure 1**). On occasion, isolated tumor cells located close to the front of proliferation were intensely stained, but the density was less than 10% of tumor cells. Excepting for tumor cells, we found scattered cells in the tumor stroma with moderate positive reaction. These cells could be macrophages, based on their morphologic features. Squamous cell carcinoma (n=3) and adenocarcinoma (n=3) were negative for VEGF. We found no significant statistic correlation between VEGF overexpression, grading or pathological form of carcinoma.

CD34 reaction was positive in the endothelium of blood vessels, some fibrocytes, and interstitial cells of the smooth muscle layer. In the tumor area only the endothelium has been found positive, and this specificity allowed us to calculate MVD. All malignant cells and non-malignant cells of the stroma were largely negative. Close to the normal urothelium we found many small blood vessels, orderly arranged, with visible lumen. The density of vessels significantly increased close to dysplastic urothelium, and vessels developed branches and tip cells between tumor cells. In some cases we noticed the immature vessels and tip cells that have the tendency to





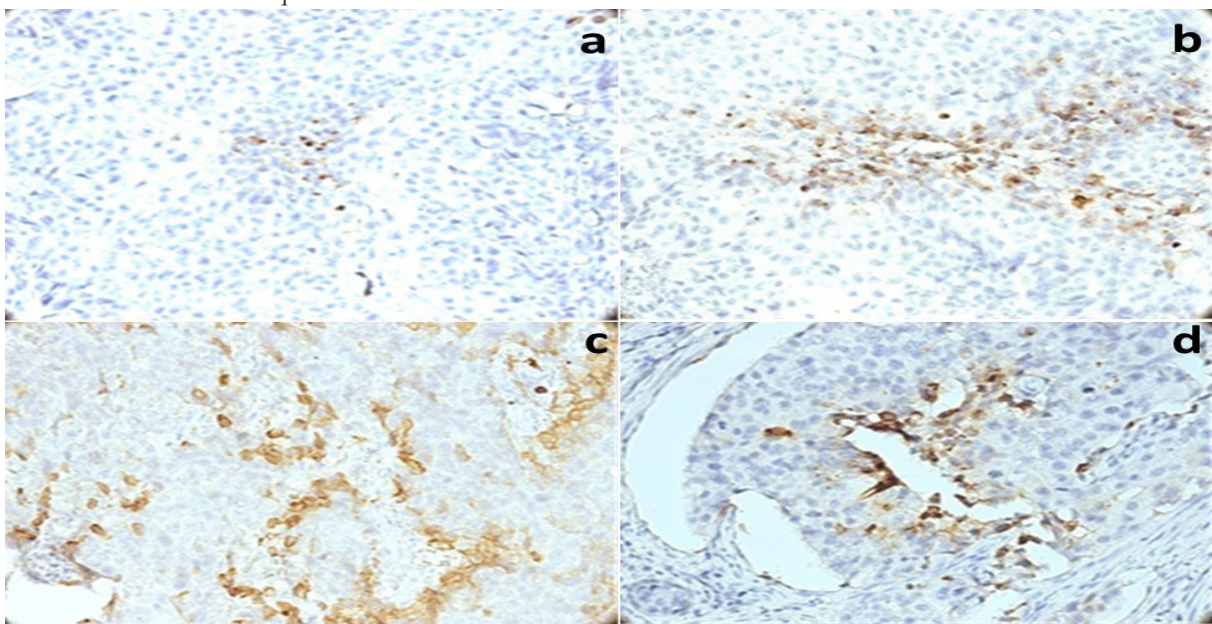
**Figure 1.** CD34 positive blood vessels. Note the vascular invasion (a). Numerous small blood vessels close to the front of proliferation (b). Immature/intermediate CD34-positive blood vessels. Note the lumen that is narrow or even not visible (c). High density of small blood vessels (d). Magnification x400.

surround small groups of tumors cells, which eventually remain included in the lumen (**Figure 2**).

Blood vessels from tumor area were irregular, with small diameter, narrow lumen or even not visible. All these vessels had immature characters, without perivascular cells. Peritumor blood vessels were larger, with thin wall. Vascular invasion was easy detected on slides stained for CD34 in 14 cases, the rate being significantly higher than that on routine stained preparations (14 versus 8). MVD in the tumor area was significantly lower in cases of urothelial carcinoma with extensive necrosis and in well-differentiated areas of squamous cell carcinoma.

In the cases with adenocarcinoma, we did not find significant differences between vessels from tumor and peritumor areas.

In urothelial invasive carcinoma MVD values calculated based on Weidner's method ranged from a minimum of 11.2 to 47.9 high power field, with an average of 28.6. We found no correlation between MVD, VEGF and conventional parameters of prognosis. On the other hand, high values for MVD correlated with blood vessel invasion and grading.



**Figure 2.** Immunohistochemical expression of VEGF in invasive urothelial carcinoma. Scattered positive macrophages in the stroma (a). Focal positive tumor cells (b). Positive reaction in tumor cells at the interface with tumor stroma (c). Strong VEGF-positive tumor cells but restricted to less than 10% (d). Magnification x400.

## DISCUSSION

More than 25 years ago Folkman demonstrated that tumors cannot survive, grow or metastasize without developing their own blood vessels by tumor angiogenesis [3]. It would seem only reasonable that an increased secretion of VEGF by the tumor cells would correlate with an increased intratumoral MVD, and a greater aggressiveness of the tumor. However, the results regarding the correlation between MVD and solid tumor prognosis reported by different authors were controversial, but there might have been a possible methodological error. This issue was solved by Weidner who standardized the techniques for counting the blood vessels to assess intratumoral MVD [4].

Bladder cancer is one of the most frequent malignant tumors of the urinary system, and is usually accompanied by both local invasion and metastasis in the moment of diagnosis [5].

As the angiogenesis is the promoter of tumor growth and metastasis, the antiangiogenic therapy became the most promising anticancer therapy. VEGF is the most powerful angiogenic substance and is expressed by normal cells, and overexpressed by tumor cells. Our results showed a moderate to low heterogeneous expression in only 13.33% of the invasive urothelial carcinoma cases. Only less than 10% of isolated tumor cells in the proliferation front overexpressed VEGF. Consequently, we draw the conclusion that there is no correlation between VEGF expression and tumor stage or recurrence. In similar conditions Bamias, found that VEGF was not correlated with MVD levels [6]. MVD levels  $\geq 47$  (assessed with CD105 antibody) were associated with longer progression-free survival after chemotherapy, hence MVD, but not VEGF, could be a useful indicator of relapse in high risk urothelial cancer cases that underwent adjuvant chemotherapy. Inoue conducted a study to assess the prognostic value of angiogenesis factor expression (MVD, VEGF, bFGF, IL8) in invasive transitional cell carcinoma of the bladder treated with neoadjuvant chemotherapy, and radical cystectomy [7]. In the pretreatment biopsy specimen VEGF expression and MVD were statistically correlated with recurrence. After chemotherapy VEGF proved to be a better predictor of recurrence than MVD and clinical stage in invasive urothelial carcinoma. The authors hypothesize that the relative overexpression of VEGF observed within the residual tumor after chemotherapy may reflect the clonal selection that allows only the tumor cells that express high levels of VEGF to survive.

Stavropoulos and colleagues found no correlation between both VEGF and MVD with any clinicopathological features, recurrence or progression in superficial primary bladder tumors), concluding that VEGF is not efficient in predicting recurrence or progression [8]. Also the authors conclude that MVD may help to predict progression in high grade patients, but not as an independent prognostic factor. Other authors while investigating the expression of VEGF and its receptors VEGFR1 and 2 in non-invasive and muscle invasive bladder cancers, found an association between these

markers and disease stage and recurrence, even if it is not statistically significant [9].

VEGF promotes angiogenesis by stimulating the proliferation and differentiation of endothelial cells, via stimulating the activation of VEGFR-2. In an experimental study, Davis used DC101, a murine specific VEGFR blocking antibody to assess its antiangiogenic effect on bladder tumors growing in nude mice [10]. In the control tumors MVD was higher at tumor periphery, where was the highest concentration of VEGFR-2 also. In this zone the angiogenesis was considered more active due to the presence of numerous smaller blood vessels. After DC101 therapy, surprisingly, MVD measured by CD105 did not decrease, in fact CD105 vessels appeared to accumulate in the tumor cores after therapy, in parallel with increased VEGFR-2 expression. The authors concluded that the results are due to increased hypoxia, and CD105 positive vessels are relatively refractory to VEGFR blocking antibody. Other authors found VEGFR expression negative in the urothelium and intensely positive in stromal blood vessels in both micropapillary urothelial carcinoma and invasive urothelial carcinoma [1]. As a consequence, they concluded that there is no antitumor effect expected for VEGFR inhibitors.

Angiotensin II type 1 receptor (AT1R) expression and high MVD correlate with early intravesical recurrence in patients with non-muscle-invasive bladder cancer [11]. AT1R antagonists (candesartan) inhibit vascular endothelial growth factor (VEGF) production and dramatically decrease lung metastasis of renal cancer by inhibiting tumor angiogenesis [12]. AT1R could be a molecular target in bladder cancer therapy.

Besides the inhibitors described above, there are also other substances that could target the new vessels, such as BAI-1 brain-specific angiogenesis inhibitor-1 [13]. BAI-1 has a strong expression in normal bladder mucosa and is negatively correlated with the expression of VEGF, with MVD and tumor stage. The authors suggest that BAI-1 may be involved in the negative regulation of microvascular proliferation, at least in bladder transitional cell carcinoma.

Tumor angiogenesis and its onset mechanisms depend not only on the tumor type, but also on the tumor microenvironment [14]. TSP-1 an extracellular matrix glycoprotein and a potent inhibitor of angiogenesis, was found to be downregulated in bladder tumors that change from an antiangiogenic to an angiogenic phenotype [15]. The authors found a positive correlation between TSP-1 stromal expression, MVD ( $p=0,031$ ) and VEGF expression ( $p=0,001$ ) in larger tumors ( $>3\text{cm}$ ) organ confined.

Microvessel density (MVD) evaluated on slides stained with CD31, CD34, CD105, or for von Willebrand factor, represents a measure of tumor angiogenesis and is used as a prognostic indicator.

In our study we used CD34 for intratumoral and peritumoral blood vessels identification, and for calculating MVD. We found that in invasive bladder cancer MVD was between 11.2 to 47.9 / high power field (average 28.6). The blood vessels in the tumor area and in the proliferation zone were immature or intermediate.



In our study high values for MVD correlated with blood vessel invasion and grading. Similar results were reported by Canoglu [16]. They described a correlation between MVD and tumor grade, stage and prognosis. High MVD was associated with the risk of clinical progression in both superficial and invasive bladder carcinomas.

Bochner found that MVD was associated with disease progression in patients with organ-confined tumors, muscle invasive tumors, or tumors that spread to regional lymph nodes [17]. In the same year Jaeger described a correlation between intratumor MVD and the risk of occult metastasis in patients with invasive bladder carcinomas [18]. Other authors reported that there was no relationship between MVD and tumor grade or stage, but high MVD was associated with a worse prognosis, and concluded that MVD is an independent prognostic marker in invasive bladder cancer [19],[20].

The results were not the same in studies about urothelial carcinoma of the bladder conducted by Hawke [21]. They found that although there was a correlation between tumor MVD and survival, it was not statistically significant hence the assessment of tumor MVD in urothelial carcinoma of the bladder is of little clinical importance. The same results were reported by Dinney: MVD was not a prognostic marker for T1 transitional cell carcinoma [22]. Other authors suggest that the prognostic significance of neovascularization is better assessed by vascular area and shape related morphometric characteristics, whereas MVD becomes influential only with respect to overall survival of patients with muscle-invasive bladder tumors [23].

In another study that investigated hypoxia inducible factor 1 alpha (HIF-1), another proangiogenic factor in voided urine samples in bilharzial and non-bilharzial bladder cancer versus benign bladder tumor the authors also calculated intratumor MVD using CD34 antibody [24]. Even if there was a statistically significant difference between benign and malignant tumors in regard with HIF-1 positivity rate ( $p < 0.001$ ), and MVD had a higher score in the malignant tumors (70% versus 0% in the benign tumors) the authors found no significant relationship between HIF-1, and MVD on one hand, and stage and tumor grade on the other hand. Hypoxia also triggers the endothelin axis that has a direct effect on MVD in tumor area. In invasive bladder cancer ET1 expression correlates with MVD in organ confined tumors [25]. In these cases, the authors obtained a surprising result: a better prognosis for patients with upregulated MVD.

## CONCLUSION

Based on our results, we conclude that VEGF is not an efficient target for therapy in patients with invasive tumors of the urinary bladder, as only 13.3% overexpressed VEGF at protein level. No correlation was found with clinic-pathological parameters, and VEGF

cannot be taken into account as individual prognostic marker or to predict the response to specific therapy with bevacizumab. Immunoreaction for CD34 is excellent to detect vascular invasion that significantly increases results obtained on routine stained slides. MVD is a useful tool for prognosis and we found correlation with vascular invasion and grading. Immature and intermediate blood vessels could be attractive targets for antivasular therapy, but further studies are necessary in this field.

## ACKNOWLEDGEMENTS

The authors are grateful to Patricia Berzava and Ciprian Onica for their excellent technical assistance. We thank to colleagues from Arad Department of Pathology, because they kindly provided archive paraffin blocks for this study. The work was financially supported by P III-C5-PCFI-3, 2017/2018-UROVESSELS grant of Victor Babes University of Medicine and Pharmacy.

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