

# MICROVESSEL DENSITY IN UROTHELIAL TUMORS OF THE UPPER URINARY TRACT

Flavia Baderca<sup>1</sup>, Rodica Lighezan<sup>1</sup>, Marius Raica<sup>1</sup>, Alis Dema<sup>2</sup>, Aurora Alexa<sup>1</sup>

## REZUMAT

**Introducere:** Densitatea vasculară a fost studiată în numeroase tumori maligne, în numeroase studii demonstrându-se relația directă dintre angiogeneză și progresia tumorii, răspunsul la tratament și supraviețuire. În acest studiu, am încercat să cuantificăm densitatea vasculară din stroma carcinoamelor uroteliale, atât la nivelul tumorii cât și în ariile din jurul acesteia, și apoi să o corelăm cu stadiul și gradul tumorii. **Material și metodă:** Studiul a fost retrospectiv și a inclus 28 de pacienți cu tumori uroteliale de tract urinar superior. Secțiunile la parafină au fost colorate cu hematoxilina-eozină. Secțiuni suplimentare au fost colorate imunohistochimic cu anticorpi anti-CD31 și anti-CD34, considerați markeri pentru endoteliu, iar microdensitatea vasculară a fost calculată prin două metode: valoare medie și valoare maximă (hot-spot). **Rezultate:** În acest studiu a fost evidențiată o strânsă corelație între microdensitatea vasculară și stadiul și gradul tumorii, așa încât angiogeneza poate deveni un bun indicator de prognostic tumoral. La nivelul stromei carcinoamelor uroteliale de tract urinar superior, au fost evidențiate vase sangvine mature și imature, aspect ce nu este prezent în carcinoamele vezicii urinare. În cuantificarea densității vasculare, considerăm că trebuie luate în calcul doar vasele sangvine cu perete imatur, datorită importanței lor în progresia tumorii și în dezvoltarea metastazelor. **Concluzii:** În studiul nostru, valoarea medie a densității vasculare calculată pentru ariile tumorale a fost direct proporțională cu stadiul și gradul tumorii, spre deosebire de ariile peritumorale, unde valoarea medie a microdensității vasculare a fost mai mare în tumorile T<sub>a</sub> și T<sub>1</sub> decât în tumorile T<sub>2</sub> și T<sub>3</sub>. Studiul nostru demonstrează o corelație strânsă între microdensitatea vasculară de la nivelul ariilor tumorale și gradul și stadiul tumorii.

**Cuvinte cheie:** tumori uroteliale, imunohistochimie, microdensitate vasculară

## ABSTRACT

**Objective:** Microvessel density (MVD) has been studied in a number of neoplasias, and apparently, there is a relationship between angiogenesis and tumor progression, response to treatment, and outcome. The purpose of this study was to determine the microvessel density in tumors represented by the urothelial carcinomas and to correlate it with tumor stage and grade. **Material and methods:** We retrospectively investigated 28 patients with urothelial neoplasms of the upper urinary tract, represented by the following subtypes of urothelial carcinomas: papillary, infiltrative and papillary-infiltrative. Step sections were stained with routine haematoxylin-eosin method. Additional slides were immunohistochemically stained with anti-CD31 and anti-CD34 antibodies, considered specific markers for endothelium. The microvessel density was estimated using the "hot-spot" method and the mean value. **Results:** Results revealed a strong relationship between microvessel density and the tumor stage and grade. In this instance, angiogenesis becomes predictive for the stage, and consecutively, for prognosis. In tumor area, we noticed the presence of mature as well as immature blood vessels, aspect that is not mentioned for bladder tumors. We believe that only immature vessels must be counted because they have highest importance in progression and developing of metastasis. **Conclusions:** In our study, mean vessel density in tumor area increased with tumor stage, while in the area around tumor only mean values of MVD were higher, in lower tumor stage T<sub>a</sub> and T<sub>1</sub>. There was a strong correlation between mean vessel density and tumor stage and grade.

**Key Words:** urothelial tumor, immunohistochemistry, microvessel density

## INTRODUCTION

Microvessel density (MVD) has been studied in many malignant tumors, and apparently, there is a relationship between angiogenesis and tumor progression, response to treatment, and outcome.

There is a strong relationship between the microvessel density as a quantitative aspect of tumor vasculature, and tumor progression, in other words, tumor angiogenesis seems to be a good predictor of progression and metastasis.<sup>1</sup> Moreover, demonstration of an active angiogenesis in malignant tumors could represent a base for the relatively new introduced anti-angiogenic therapy.

Urothelial tumors of the upper urinary tract represent less than 5% of the renal tumors, excepting the areas with Balkan Nephropathy, where they exceed 9%. In spite of the fact that more than 95% of tumors of the upper urinary tract are urothelial, the morphologic and immunohistochemical profile overlaps only in part with those reported for similar tumors developed in the urinary bladder, which

<sup>1</sup>Department of Histology, <sup>2</sup>Department of Pathology, Victor Babes University of Medicine and Pharmacy, Timisoara

Correspondence to:  
Flavia Baderca, MD, Victor Babes University of Medicine and Pharmacy, 2 E. Murgu 2 Sq., 300041, Timisoara, Romania, Tel. +40256 204476  
Email: flaviabaderca@yahoo.com

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are well known nowadays. Because of the reduced incidence of patients with urothelial tumors of upper urinary tract, relatively few data are available about their natural evolution and behavior.

In urothelial tumors of the urinary bladder, MVD and its relationship with progression were better studied, in spite of some conflicting results. Some authors revealed the correlation between the number of blood vessels from the tumor area and bad prognosis. Others showed favourable prognosis in the case of angiogenesis developed within or close to the tumor-associated lymphoid infiltrate. It is thought that MVD associated to the inflammatory infiltrate correlates with long-term survival.<sup>2</sup>

To our knowledge, only few papers were published so far about the microvessel density in urothelial tumors of the upper urinary tract.<sup>3-5</sup>

The aim of the present study was to determine the MVD in tumor area and in areas around the tumor and to identify a correlation between the MVD and tumor stage or grade.

## MATERIALS AND METHODS

### Specimens

The study retrospectively investigated 28 patients aged between 47 and 68 years, admitted in the County Hospital of Timisoara, which gave their consent for the specimen analysis. The patients presented urothelial neoplasms of the upper urinary tract, represented by urothelial carcinomas with three subtypes: papillary, infiltrative and papillary-infiltrative. The surgical technique performed in all patients was nephro-ureterectomy, so in each case specimens were taken from the tumor, kidney and ureter. The localization of primary tumor is shown in Table 1. Specimens were fixed in 4% buffered formalin, embedded in paraffin, and step sections were stained with routine hematoxylin-eosin method.

**Table 1.** Sites of primary tumors.

Tumor site	Number of cases	%
Calyces	4	14
Renal pelvis	5	18
Calyces and renal pelvis	7	25
Ureters	8	29
Renal pelvis + ureters	4	14

### Immunohistochemistry

Additional slides were immunohistochemically stained, using the three step labeled streptavidin-biotin-immunoperoxidase technique (LSAB2, DAKO,

Glostrup, Denmark). After endogenous peroxidase inhibition, antigen retrieval, the sections were incubated with the first step antibodies: anti-CD31 (monoclonal, clone JC70A) and anti-CD34 (monoclonal, clone QBEnd10). Thereafter, the slides were washed in PBS and consecutively reacted with a labeled streptavidin-biotin system. The final reactive product was visualized in brown with DAB as chromogen. The slides were counterstained with Mayer's hematoxylin. CD31 also known as PECAM-1 is a glycoprotein found on endothelial cells and various hematopoietic cells. This molecule is involved in cell adhesion and mediates the transmigration of leucocytes from blood vessel lumen into the extra vascular space. CD34 is a transmembrane glycoprotein present on endothelial cells and hematopoietic stem cells.<sup>6,7</sup> Anti-vimentin (clone V9) antibody was used as a marker of the optimal fixation and embedding procedures. The external positive control was represented by slides from a capillary hemangioma. As intern positive control, we used immunolabeling of endothelial cells of the glomerular capillaries. As negative control, it was used a nonspecific immunoglobulin, provided by the manufacturer (DAKO, Denmark), performed on slides from the same cases.

### Microvessel density

The microvessel density (MVD) was estimated by counting the blood vessels in the tumor and the peritumor area, using the "hot-spot" method and the mean value. The hot spot method consisted in counting blood vessels on the microscopic field with the highest density of vessels, at high magnification (x200). Mean values of MVD were calculated after examining 10 consecutive microscopic fields in the tumor area and around it.

Images were acquired using a Nikon Eclipse 600 microscope, fitted with Coolpix 950 digital camera. The camera was connected to a computer loaded with Lucia G soft for microscopic image analysis. Selected areas were stored as JPEG files (1200x1600 pixels), and there were counted only blood vessels without smooth muscle cell layer. Accurate assessment of MVD in papillary tumors is not possible according some authors,<sup>8</sup> and therefore, only solid or mixed solid/papillary tumors were included in the study (n = 26).

Continuous data were expressed as mean  $\pm$  SD and categorical data as percentages. Results were analyzed for statistical significance by Student t test using the InStatGraphPad program on a HP personal computer. Differences were considered significant at  $p < 0.05$ .

## RESULTS

Two independent pathologists reviewed the hematoxylin-eosin stained slides in order to assess the histopathological diagnostic. The histological form of urothelial carcinomas, the degree of differentiation (G) and associated lesions were noticed for all the cases. Most of the patients had tumors in stage T<sub>2</sub> or T<sub>3</sub> (77%), and the distribution of cases, considering the postoperative stage (TNM stage) is presented in Table 2.

**Table 2.** Tumor staging after surgery.

TNM	Number of cases (n)	%
T <sub>a</sub> N <sub>0</sub> M <sub>0</sub>	1	4
T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	5	19
T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	13	50
T <sub>3</sub> N <sub>0</sub> M <sub>0</sub>	5	19
T <sub>3</sub> N <sub>1</sub> M <sub>0</sub>	1	4
T <sub>3</sub> N <sub>2</sub> M <sub>0</sub>	1	4

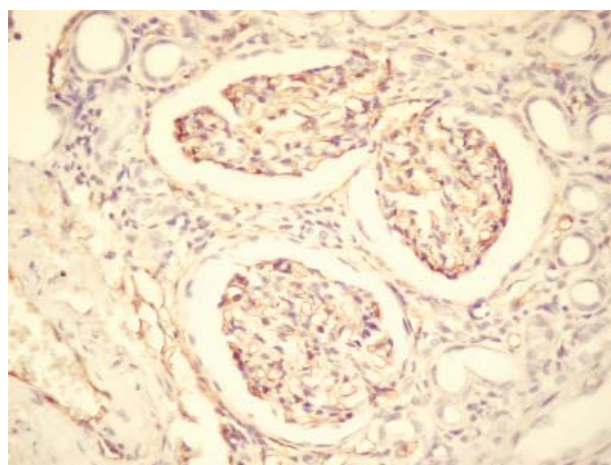
The urothelial carcinomas with the capacity to invade and metastasize were divided into a small group (n = 6) with features of low cytologic grade (G<sub>1</sub>) and a larger group (n = 20) of high cytologic grade (G<sub>2</sub> and G<sub>3</sub>), as shown in Table 3. This classification was made depending upon the degree of nuclear anaplasia and some architectural abnormalities.

**Table 3.** Tumor grading.

Grade	Number of cases (n)	%
G <sub>1</sub>	6	23
G <sub>2</sub>	12	46
G <sub>3</sub>	8	31

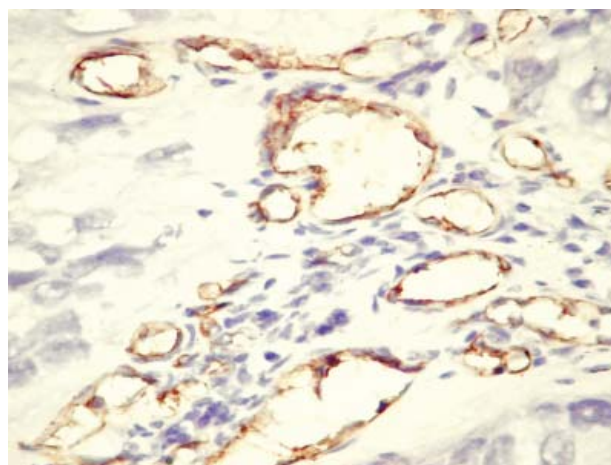
Blood vessels belonging to the tumor microvasculature have been visualized with anti-CD31 and/or anti-CD34 antibodies, the highest sensibility of immunolabeling being provided by anti-CD34 antibody and the highest specificity by anti-CD31 antibody. (Fig. 1) The reaction product was present in the cytoplasm of the endothelial cells. In the tumor area the vessels had a narrow lumen, and many fine ramifications. Nests of endothelial cells were also present, suggesting the progression of angiogenesis.

In order to determine the mean value of MVD, we counted all blood vessels positive for endothelial markers on 10 consecutive microscopic fields at high power magnification (x200), from both tumor and peritumor area. The blood vessels with muscular media were not counted and we did not examine tumor areas with necrosis and hemorrhage.



**Figure 1.** Immunohistochemical staining showing positive expression for CD34 in endothelium-lined normal and tumor vessels.

The maximum value of MVD was determined with the “hot-spot” method, on three most vascular fields (x200). The highest value of the vessel count, out of these three fields, was designated as the maximum value of microvessel density. In the tumor area, mature as well as immature blood vessels were present. (Fig. 2)



**Figure 2.** Mature and immature vessels positive for anti-CD34 antibody.

Table 4 presents the relationship between the MVD of tumor area and tumor stage. The results were statistically significant for mean and maximum values of MVD. In the present study, the range of values of blood vessels counting was statistically significant only for the tumor stages T<sub>2</sub> and T<sub>3</sub> (p < 0.05), and not significant statistically for the tumor stages T<sub>1</sub> and T<sub>a</sub>. In the tumor stage T<sub>a</sub> the vessels were absent so that there was no immunolabeling for the endothelial markers in the tumor area. In tumors with necrosis, the blood vessels were less numerous.

Around the tumor area, the count of the blood vessels from connective tissue was made on the same manner like for the tumor area (Figs. 3,4). In tumors with a rich inflammatory infiltrate, the blood vessel

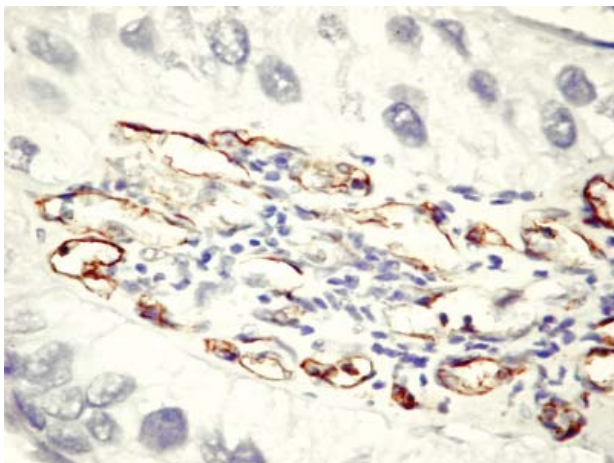
were numerous in the peritumor area. The blood vessels of tumor papillae were excluded from study. The results are presented in Table 5.

**Table 4.** The microvessel density in the tumor area expressed as mean value and hot-spot.

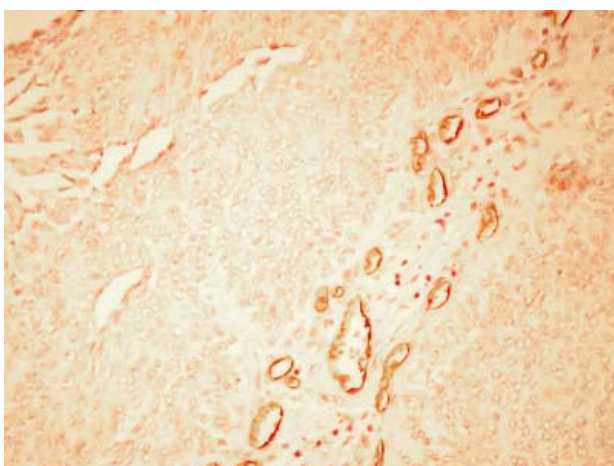
T/MVD	Mean value	Hot - spot	Range of values
T <sub>1</sub>	9.70	16.00	6.00 - 16.00
T <sub>2</sub>	23.80	34.00	15.00 - 34.00
T <sub>3</sub>	37.00	50.00	24.00 - 50.00

**Table 5.** Microvessel density in the areas around the tumor.

T/MVD	Mean value	Hot-spot	Range of values
T <sub>a</sub>	38.00	41.00	35.00 - 41.00
T <sub>1</sub>	20.70	25.00	12.00 - 25.00
T <sub>2</sub>	11.40	15.00	6.83 - 15.00
T <sub>3</sub>	18.77	24.00	13.00 - 24.00



**Figure 3.** Blood vessels in the tumor stroma positive for anti-CD34 antibody.



**Figure 4.** Distribution of blood vessels in the peritumor area, intensely positive for CD31, the specific marker of endothelium.

In the connective tissue around the tumor, only the mean values of the microvessel density were higher in

the tumor stages T<sub>4</sub> and T<sub>1</sub> compared with tumor stages T<sub>2</sub> and T<sub>3</sub>. The highest value of MVD respected this relationship. If we search for the relationship between the range values of the MVD on ten consecutive microscopic fields and tumor stage, as the range values decreased the tumor stage increased.

The relationship between microvessel density and the tumor grade was calculated only for the tumor area. (Table 6) For a better interpretation, we considered the tumor grades G<sub>3</sub> and G<sub>4</sub> only one group. Both the mean values and the highest values of the MVD on ten consecutive microscopic areas were significantly correlated with tumor grade.

**Table 6.** Microvessel density and tumor grade.

G/MVD	Mean value	Hot-spot
G <sub>1</sub>	6.10	10.00
G <sub>2</sub>	23.76	36.50
G <sub>3</sub>	33.07	41.80

## DISCUSSIONS

In urologic oncology, researches on angiogenesis discovered that urothelial carcinomas may induce angiogenesis that significantly contributes to their malignant phenotype.<sup>9</sup> The angiography with fluorescein revealed accumulation of blood vessels in papillary tumors and CIS in comparison with normal bladder, confirming that these tumors are highly vascular.<sup>10</sup>

The growth of the tumor over 2 mm in diameter strongly depends on the formation of new blood vessels, and urothelial tumors make no exception from this rule. The increase of the MVD together with the tumor stage is not surprising, because the stage depends in part on the diameter of the tumor. This observation is supported by the knowledge that tumor size is proportional with number of vessels present in the tumor. Some authors failed to demonstrate such a correlation. Discrepancies may be attributed to the methodology: “hot-spot” or mean value, to the microscopic field (from x100 to x400), to the type of antibodies (factor VIII, CD31 or CD34) and to the small number of patients included in the study.

Areas with the highest density of blood vessels seem to be very important, because it may represent a route used by malignant cells to metastasize. Microvessels with large lumen were numerous in T<sub>2</sub> tumors. This observation supports the hypothesis of the primary initiation of angiogenesis by positive regulators that finally induce formation of large

sinusoidal spaces.<sup>11,12</sup> This moment is coincident with the transition from superficial to invasive urothelial carcinoma. The diameter of new vessels is restricted, in relation with the exponential growth of the tumor, but the increased rate of new blood vessels formation remains constant.

Only a few authors examined the relation between angiogenesis and survival in invasive urothelial carcinomas, and in superficial tumors this aspect is uncertain. It seems that factors that mediate angiogenesis in superficial and respectively, invasive tumors are different. In invasive tumors the increase of MVD is associated with low survival.<sup>13,14</sup> The larger the microvessel is, the higher are the chances for malignant cells to pass in the circulation. Recent data suggested that the vascular area is the only parameter with predictive value for survival in superficial tumors.<sup>15,16</sup>

The relationship between MVD and prognosis in bladder tumors is far from being clarified. It was reported that increased MVD, associated to the inflammatory infiltrate correlates with prolonged survival.<sup>2</sup> This is why we evaluated the MVD in the tumor area and in the surrounding connective tissue.

Angiogenesis represents the formation of new blood vessels from pre-existing vasculature. Tumor angiogenesis was first defined by Judah Folkman in 1970. Data on angiogenesis in tumors of the upper urinary tract are poor. Until now, only a few articles were published on the subject. In one of them, 91 patients were included, and the authors failed to demonstrate a relationship between MVD, stage, progression, and metastasis.<sup>3</sup> It is important to emphasize that these authors used a single endothelial marker (factor VIII), that is not enough sensitive for tumor-associated vessels. As the immunohistochemical procedures refined, other authors demonstrated, using other endothelial markers, that increased MVD correlates with unfavorable prognosis.<sup>4</sup>

Our results revealed a strong relationship between the MVD and the tumor stage and grade. In this instance, angiogenesis becomes predictive for the stage, and consecutively, for prognosis. In tumor area, we noticed the presence of two types of vessels: mature and immature, aspect that is not mentioned for bladder tumors. Immature vessels are important for the tumor progression and developing of metastasis. Mechanisms involved in tumor progression are incompletely understood.<sup>17</sup> For sure, tumor angiogenesis is not the only process responsible for progression, but considering the number of microvessels in T<sub>2</sub> and T<sub>3</sub> tumors, it occupies a central place.

The endothelial cells from the immature vessels

are main target for the antiangiogenic therapy, which tend to bring the tumor in a dormant state.<sup>18,19</sup> Tumor growth is angiogenesis-dependent, and in angiogenesis VEGF plays a central role, so that VEGF is a promising target for therapeutic intervention. The majority of angiogenesis inhibitors block the VEGF.<sup>20</sup> The angiogenesis inhibitors are considered a new class of drugs, used in association with the conventional chemotherapy. Both chemotherapy and antiangiogenic therapy depend on endothelial cell apoptosis.<sup>21</sup>

## CONCLUSIONS

In our study, there was a strong correlation between mean vessel density and tumor stage and grade. In tumor area, mean vessel density increased with tumor stage, while in the area around tumor, only mean values of MVD were higher, in lower tumor stage: T<sub>a</sub> and T<sub>1</sub>.

Our findings suggest an association of advanced urothelial carcinomas with an increased angiogenesis. The majority of new formed vessels were immature-type.

In view of current efforts to study antiangiogenic drugs, as a treatment of different neoplasias, we believe that these patients could benefit from anti-angiogenic therapies.

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