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Source / Izvornik: **Collegium Antropologicum, 2011, 35, 1071 - 1079**

Journal article, Published version

Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

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# Relationship Between Tumor Vascularity and Vascular Endothelial Growth Factor as Prognostic Factors for Patients with Neuroblastoma

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## ABSTRACT

*Although the role of angiogenesis in tumor progression and response to treatment is generally well-characterized, for neuroblastomas clinical data regarding the contribution of angiogenesis and its predictive capacity remain unclear. The aim of this study was to evaluate whether tumor vascularity in the combination with expression of vascular endothelial growth factor (VEGF) represent prognostic factors for patients with neuroblastoma. Immunohistochemistry using anti-CD34 and anti-VEGF antibodies was used to analyze paraffin-embedded primary tumor tissues from 56 patients diagnosed with neuroblastoma. Tumor vascularity was estimated by calculating the tumor vascular volume fraction (TVVF), and VEGF expression was determined using semi-quantitative scoring. Statistical analyses including multivariate analysis were performed and compared with these two factors. Tumor vascularity had impact on survival of high VEGF expression neuroblastoma patients. Combination of high VEGF expression and TVVF value  $\leq 5\%$  was independent predictor of overall survival ( $p$ -value = 0.0041, odds ratio (OR) (95%CI)=8.67 (1.99–37.69) by the Cox proportional hazards model). This study revealed for the first time a group of extremely high-risk neuroblastoma with both high VEGF expression and poor vascularity. For these patients reduced rates of survival were observed (37% vs. 92.5%) ( $p < 0.0001$ ). These patients did not experience a significant improvement following hematopoietic stem cell transplantation, and could be candidates for receiving novel therapies. These results indicate the importance of the mutual relationship between tumor vascularity and VEGF, because it gives better insight into the prognosis of patients with neuroblastoma.*

**Key words:** neuroblastoma, tumor angiogenesis, vascular endothelial growth factor, hypoxia, survival

## Introduction

Angiogenesis plays an important role in the progression and metastasis of many types of malignant tumors. For neuroblastoma, this pediatric malignancy is associated with a broad spectrum of clinical behavior, and the extent of angiogenesis present appears to affect the tumor's phenotype<sup>1</sup>. The clinical heterogeneity and embryonal origin of this disease also suggest that regulation of neovascularization is complex<sup>2</sup>. Various studies have confirmed that a positive correlation exists between tumor microvascular density and an aggressive tumor pheno-

type in cases of neuroblastoma<sup>3,4</sup>. However, other studies did not find that vascular parameters of neuroblastoma predicted patient survival rates<sup>5,6</sup>, and even found that low vascular density was associated with short survival<sup>7</sup>.

The prognostic value of microvascular density has been examined for different types of cancer. Most of these studies have reported a positive correlation between tumor vascularity and tumor recurrence<sup>8–20</sup>. However, other reports did not find a positive association be-

tween increased tumor vascularity and reduced survival, which brings into question the clinical utility of tumor angiogenesis<sup>21–27</sup>.

VEGF is an endothelial cell-specific mitogen that is one of the most important stimulators of tumor angiogenesis and tumor growth<sup>28,29</sup>. Furthermore, previous studies have demonstrated that expression of VEGF correlates with a high-risk phenotype in neuroblastomas<sup>28,30–33</sup>. Correspondingly, our previous study identified VEGF expression as an important biological marker for neuroblastomas and it could be used to identify high-risk neuroblastoma patients in combination with tumor stage and other relevant risk factors<sup>34</sup>.

Given that the mechanisms of tumor angiogenesis are still enigmatic, and relationship between VEGF and tumor angiogenesis in neuroblastoma still unexplored, tumor vascular volume fraction (TVVF) was calculated in relation to VEGF expression for cases of neuroblastoma. Furthermore, these factors were evaluated in relation to the prognosis of the neuroblastoma patients analyzed.

## Materials and Methods

Neuroblastoma tissue samples (n=56) were obtained from the archives of the Institute of Pathology, School of Medicine, University of Zagreb, Croatia for patients who had undergone treatment at the Department of Hematology and Oncology of Children's Hospital Zagreb (Zagreb, Croatia) between 1995 and 2008. Clinical staging was classified according to The International Neuroblastoma Staging System (INSS)<sup>35,36</sup>. Patients were divided into two groups based on their stage of disease. The »low stage« group included patients with stage 1, 2, or 4s neuroblastoma, and the »high stage« group included patients with stage 3 or 4 neuroblastoma. Histopathological grading was classified according to the Shimada System and Shimada Age-based Pathologic Classification<sup>37,38</sup>. All of the histological samples were reevaluated and received new grading (SS). Patients with stage 1, 2, or 4s disease (N=19) were treated with surgery alone, or surgery and moderate-dose chemotherapy. Alternatively, stage 3 and stage 4 patients (N=37) were treated with surgery in combination with intensive, multi-agent chemotherapy with or without radiotherapy and/or metaiodobenzylguanidine (MIBG) therapy. In addition, 14 patients with advanced disease, and 3 patients with localized disease with N-myc amplification, received megatherapy (i.e., myeloablative chemotherapy) followed by autologous or allogeneic hematopoietic stem cell transplantation. Since hematopoietic stem cell transplantation for high-risk patients was started in 1999, high-risk patients either received, or did not receive, stem cell transplantation (Table 1).

Following surgery, specimens were fixed in 4% buffered formalin for at least 24 h, then were embedded in paraffin, and stored at room temperature until sectioned. Serial sections (4–5 µm) were cut and adhered onto microscope slides. Paraffin was removed from the sections using xylene and the samples were rehydrated.

**TABLE 1**  
PATIENT CHARACTERISTICS

Characteristics	No. patients (N)
Total number	56
Gender	
Male	35
Female	21
Age	
>18 months old	36
≤18 months old	20
Histologic subtype	
Stroma-rich	16
Stroma-poor	40
Histology	
Favourable	23
Unfavourable	33
Stage	
Low	19
High	37
Treatment	
S	3
S/CTH	32
Deceased	21

Abbreviations: S, surgery; CTH, chemotherapy; MIBGT, metaiodobenzylguanidine therapy; BMT, bone marrow transplant; RT, radiotherapy

## CD 34 immunohistochemical analysis

Murine CD34 monoclonal antibody (Dako, Glostrup, Denmark) was used at a dilution of 1:50, and was incubated with sections for 30 min at room temperature. Sections were then incubated with peroxidase-conjugated polymers linked with an appropriate secondary antibody (Envision-Detection System, Dako, Glostrup, Denmark). Antibody binding was visualized using 3, 3'-diaminobenzidine (DAB) chromogenic solution. Negative controls were included and were incubated with Tris-buffered saline (TBS) instead of primary antibody.

Low power field screening of immunohistochemically stained slides was used to identify areas of highest vascularization (»hot spots«). Visible necrosis and calcifications were excluded. Image analysis was performed on 3 medium power fields using a 10x objective, and these fields were imaged using an Olympus BX microscope and Sony 3 chip video camera. Images were then converted to gray scale using a picture archiving and communication system (PACS) (ISSA, VAMSTECH, Zagreb, Croatia). TVVF was measured as the percentage of stained endothelial area (vascular area) relative to the entire analyzed area (image area). Quantitative analysis was performed using the Electronic Automated Measurement User System (EAMUS<sup>TM</sup>, DiagnomX GmbH, Germany) (<http://www.diagnomx.eu/portal/home.php>).

**TABLE 2**  
VEGF SCORING SYSTEM

Percentage of positive tumor cells	Score <sup>1</sup>	Staining intensity	Score <sup>2</sup>
<1%	0	Weak	1
1–25%	1	Moderate	2
26–50%	2	Strong	3
51–75%	3		
76–100%	4		
Total score*			
Weak 0–2			
Strong 3–7			

Total score\* = Score<sup>1</sup> + Score<sup>2</sup>

### VEGF immunohistochemical analysis

Sections were incubated in 3% hydrogen peroxide for 5 min, then with VEGF-specific mouse monoclonal IgG (dilution 1:25; Dako, Glostrup, Denmark) for 30 min at room temperature. Bound antibodies were detected using a secondary antibody detection kit (LSAB+ kit, Dako, Glostrup, Denmark) and visualized using DAB. Sections were also counterstained with Mayer's hematoxylin and dehydrated before being mounted for analysis. Negative controls were included and were incubated with Tris-buffered saline (TBS) instead of primary antibody. For positive controls, sections of colon carcinoma were used.

Staining of VEGF was semi-quantitatively analyzed using a scoring system described in Table 2. Briefly, the percentage and intensity of stained tumor cells were determined and scored. When these two sets of scores were added together, specimens were then classified as weak (0–2) or strong (3–7).

### Statistical analysis

Descriptive statistics and 95% confidence intervals (CI) were used. Data distribution was analyzed using the D'Agostino-Pearson test. According to the type of distribution, an appropriate parametric, or an equivalent non-parametric, test was performed. Spearman's coefficient of rank correlation ( $\rho$ ) was determined to assess the correlation between VEGF scores and TVVF. The cutoff value for determining low *vs.* high VEGF expression scores, as well as low *vs.* high TVVF scores, was determined from an analysis of receiver operating characteristic (ROC) curve<sup>39</sup>. The relationship between VEGF expression, tumor vascularity, gender, age, tumour localisation, stage, histology and survival was estimated by applying the Fisher's exact test. Overall survival (OS) was defined as the interval between the time of established diagnosis and patient's death. Univariate analysis of OS was performed as outlined by Kaplan and Meier<sup>40</sup>. Statistical significance of differences in survival between the patient groups was estimated using the log-rank test. The Cox proportional hazards model was used for multivariate analysis to determine independent predictors of

overall survival. Differences were considered significant at  $p < 0.05$ . Statistical analyses were performed using MedCalc version 10.4 (MedCalc Software, Mariakerke, Belgium).

## Results

Characteristics of neuroblastoma patients are listed in Table 1. The mean patient age was 35.5 months (range: 2 months to 12 years), the median patient follow-up time was 27 months (range: 1.0–180.0 months), and the overall survival rate was 62.5%. Overall survival rates were calculated at 24 months.

### VEGF expression

Immunohistochemical staining was used to detect VEGF expression (Figures 1a-c). Consistent with a previous study that also analyzed these sections, VEGF staining was detected in 54/56 (96.4%) of the tumor samples<sup>34</sup>. The distribution of VEGF scores was normal. Using ROC curve analysis, a cut off value of  $>2$  (sensitivity 100.0%, specificity 33.3%) was established, and tumors were distinguished as having high (score: 3–7) or low (score: 0–2) levels of VEGF expression. A total of 44 (78.6%) and 12 (21.4%) specimens were associated with each group, respectively (Table 3). A significant correlation between the VEGF scores and tumor stage was identified ( $P = 0.002$ ,  $\rho = 0.40$ )<sup>34</sup>.

### Tumor vascularity

CD34 immunohistochemical slides were shown on Figures 1d-e. The distribution of TVVF values was not normal. The median TVVF value was 2.5%, while the lowest and highest TVVF values were 0.016% and 11.83%, respectively. Using ROC curve analysis, a cut off value of  $\leq 5$  (sensitivity 90.0%, specificity 41.7%) was established, and tumors with high ( $>5\%$  TVVF) *vs.* low ( $\leq 5\%$  TVVF) levels of vascularity were identified. Overall, a total of 39/56 (69.6%) cases were observed to have low levels of tumor vascularity, and 17/56 (30.4%) cases exhibited high levels of vascularity (Table 3). No correlation between tumor vascularity and tumor stage was observed ( $p = 0.98$ ,  $\rho = 0.002$ ).

**TABLE 3**  
VEGF EXPRESSION AND TUMOR VASCULARITY (TVVF\*)

Category	No. Patients N (%)	Surviving/Deceased N/N
Expression score		
Low (0–2)	12 (21.4%)	12/0
High (3–7)	44 (78.6%)	23/21
Tumor vascularity		
Low (TVVF $\leq 5\%$ )	39 (69.6%)	21/18
High (TVVF $> 5\%$ )	17 (30.4%)	15/2

\*Tumor vascular volume fraction =  $100 \times$  vascular area/image area



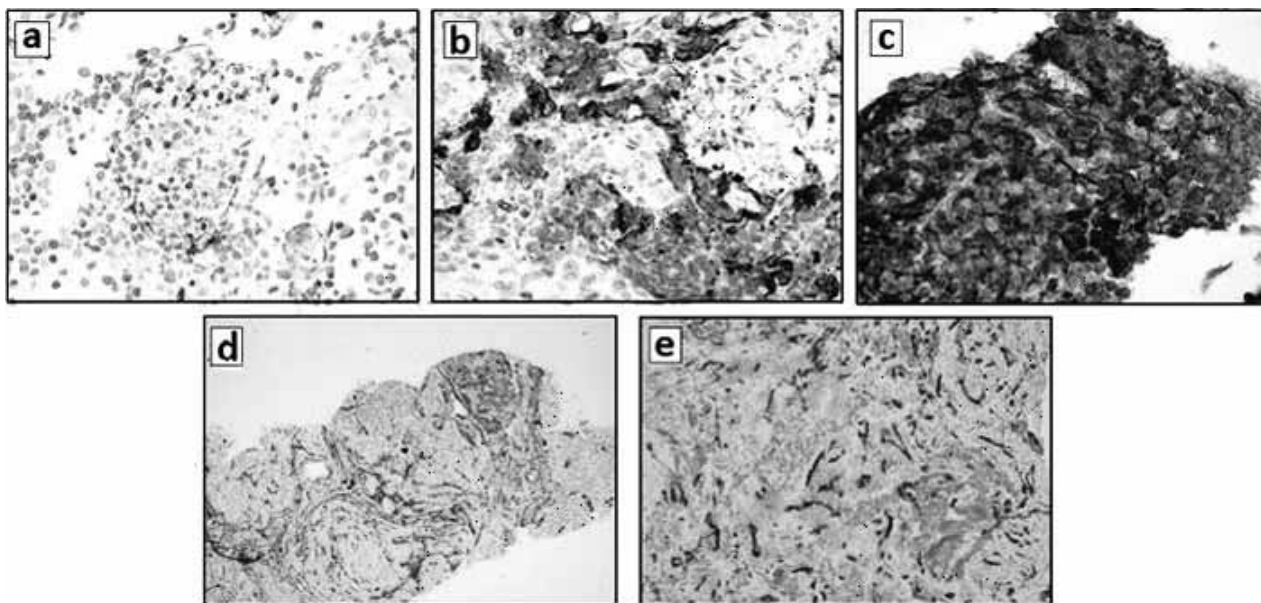


Fig. 1. VEGF and CD34 immunohistochemical staining in neuroblastoma pathohistological sections. Representative image of a section that received a) a low VEGF expression score (low grade intensity and 1–25% tumor cell positivity); b) a high VEGF expression score (high grade intensity and 25–50% tumor cell positivity); c) a high VEGF expression score (moderate grade intensity and 75–100% tumor cell positivity). (a–c) Objective = 40X; d) Immunohistochemical staining of endothelial cells in a neuroblastoma section detected using anti-CD34 antibody. (d) Objective=4X; (e) Objective=10X.

**Correlation between VEGF score and TVVF**

A significant positive correlation between VEGF scores and tumor vascularity (TVVF) was identified using Spearman’s coefficient of rank ( $\rho=0.347$ ,  $p=0.010$ ). High VEGF expression also correlated with higher TVVF values (median 2.89% vs. 1.19%), although this difference was not statistically significant according to the Mann-Whitney test.

**Survival analysis**

The overall survival rate (OSR) for patients with high vs. low levels of VEGF expression was 54.5%, and 100%, respectively. Furthermore, the tumors of deceased patients were associated with higher levels of VEGF expression compared to surviving patients ( $p=0.0046$ , Figure 2a).

For patients with low tumor vascularity (TVVF $\leq 5\%$ ) vs. high tumor vascularity (TVVF $> 5\%$ ), their OSRs were 53.8% and 88.0%, respectively. Moreover, the difference in tumor vascularity values for surviving vs. deceased patients had a p-value of 0.056 by Mann-Whitney test. For TVVF values, those of the deceased patients were lower than those of the living patients. The median values for these two groups were 1.19% and 2.83%, respectively. Furthermore, a greater number of deceased patients were associated with tumors characterized by TVVF values  $\leq 5\%$  compared to surviving patients associated with TVVF values  $> 5\%$  ( $p=0.0162$ , Figure 2b).

Kaplan-Meier survival curves were generated based on high vs. low VEGF expression (Figure 3a), and for TVVF values  $> 5\%$  or  $\leq 5\%$  (Figure 3b). Survival rates for patients with low VEGF expression ( $p=0.0053$ ) and high

TVVF values ( $p=0.0227$ ) were found to have a better outcome. Furthermore, tumor vascularity did not impact the survival of patients with low VEGF expression, since all of these patients survived.

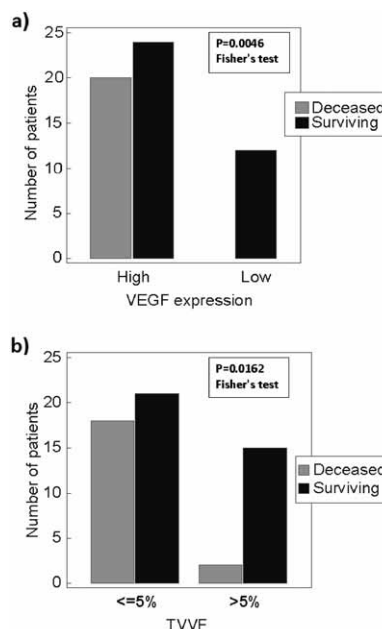


Fig. 2. Relationship between high vs. low VEGF expression and TVVF values for surviving vs. deceased patients. a) According to Fisher’s test, a p-value of 0.0046 was associated with VEGF expression levels; and b) a p-value of 0.0162 was associated with TVVF values  $>$  or  $\leq 5\%$ . Expression of VEGF and TVVF values were significant prognostic factors.

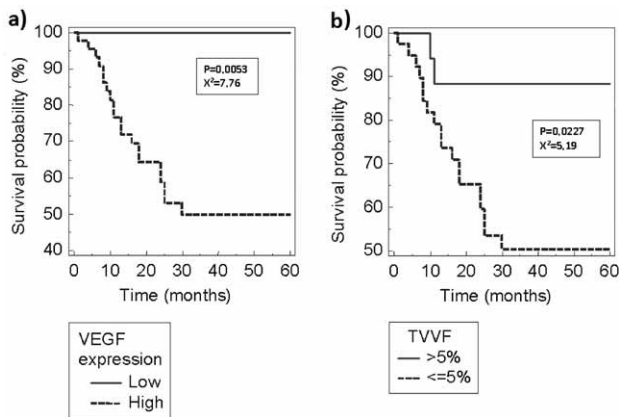


Fig. 3. Kaplan-Meier survival curves for high vs. low VEGF expression and TVVF values. a) The blue line represents patient survival associated with low VEGF expression, and the red line represents patient survival associated with high VEGF expression. b) The blue line represents patient survival associated with a TVVF value >5%, and the red line represents patient survival associated with TVVF values ≤5%. Both VEGF expression and TVVF were significant prognostic factors.

### The impact of tumor vascularity on the survival of neuroblastoma patients with high VEGF expression

For tumors with high VEGF expression and TVVF values ≤5% vs. >5%, the OSRs were 37.9% and 86.6%, respectively. Moreover, this difference was significant by Fischer's exact test ( $p=0.0034$ ) and by Kaplan-Meier analysis ( $p=0.0032$ , Figure 4). Deceased patients were associated with significantly lower TVVF values (median 1.19%) than surviving patients (median 5.02%) by Mann-Whitney test ( $p=0.0063$ ).

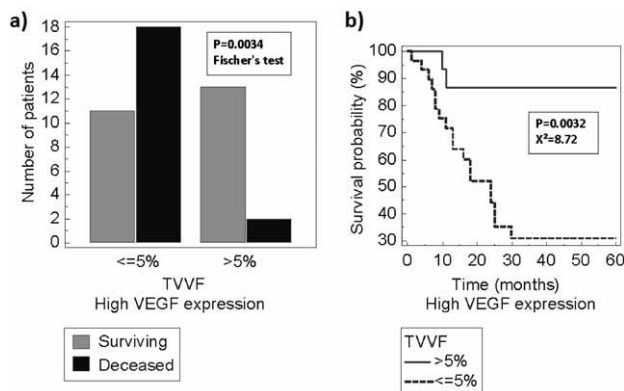


Fig. 4. The impact of tumor vascularity on the survival of patients with high levels of VEGF. a) Using Fischer's exact test, surviving (red) vs. deceased (blue) patients with high levels of VEGF were compared with respect to TVVF values (>5% vs. ≤5%); b) A Kaplan-Meier survival curve represents the patients with high levels of VEGF expression that were associated with TVVF values >5% (blue) and ≤5% (red). A significant difference in patient survival for patients with high levels of VEGF expression according to different tumor vascularity was observed.

### Correlation between VEGF score and TVVF in surviving and deceased patients

Using Spearman's coefficient of rank, TVVF values were also found to correlate with VEGF expression in surviving patients ( $p=0.003$ ), yet not for deceased patients ( $p=0.185$ ).

### High VEGF expression and low tumor vascularity as a complex variable

Survival of patients with neuroblastoma was also analyzed with VEGF expression and tumor vascularity evaluated as a complex variable (Figures 5 a,b). When patients with high levels of VEGF expression (score >2) and low vascularity scores (TVVF ≤5%) were evaluated using this approach, an OSR of 37.9% was identified, compared to an OSR of 92.6% associated with all other patients ( $p=0.000018$  by Fischer's test and  $p<0.0001$  by log-rank test).

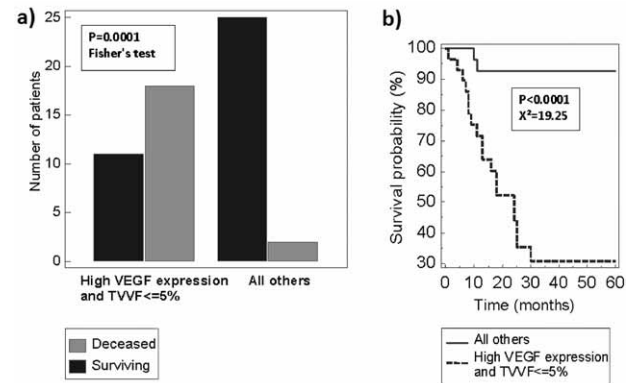


Fig. 5. The impact of VEGF expression and tumor vascularity as a complex variable on survival. a) Using Fischer's exact test, patient survival was evaluated for high VEGF expression and low TVVF values for both deceased (red) and surviving (blue) patients; b) Kaplan-Meier survival curve shows the survival of patients with high VEGF expression and low TVVF values (red line) vs. other patients (blue line). The combination of VEGF expression and TVVF values were found to be significant predictors of patient survival.

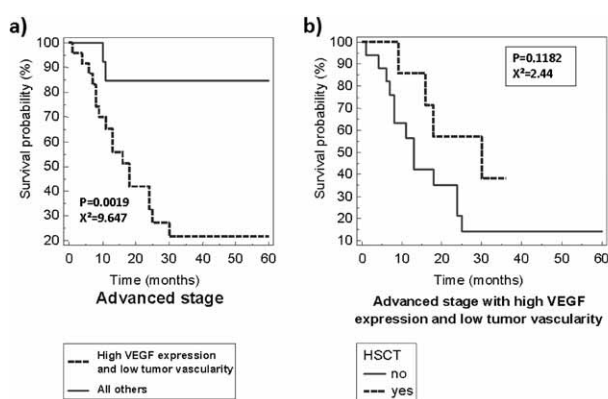
To further examine characteristics of this subset of patients, they were evaluated according to age, tumor stage, histology, and tumor localization (Table 4). As a result, a greater number of advanced stage disease patients were identified (82.8% vs. 48.1%) ( $p=0.0142$ ) that also were associated with an unfavorable histology (86.2% vs. 59.2%) ( $p=0.0334$ ). Other differences were not statistically significant. Patients with advanced stage tumors (stage 3 and 4) also had their OSR evaluated in relation to high levels of VEGF expression and low tumor vascularity as a complex variable (Figure 6a), and in relation to hematopoietic stem cell transplantation (HSCT) (Figure 6b). An OSR of 29.0% was identified in comparison with other advanced stage patients (OSR 84.6%,  $p=0.0019$ ), and HSCT was not found to improve their survival significantly ( $p=0.11$ ).

**TABLE 4**  
CLINICOPATHOLOGICAL CHARACTERISTICS OF PATIENTS WITH HIGH LEVELS OF VEGF\* AND LOW LEVELS OF TUMOR VASCULARITY\*\* EVALUATED AS A COMPLEX VARIABLE

Characteristics	High VEGF &		All other patients		Fischer's test p-value
	Low tumor vascularity		N	(%)	
	N	(%)	N	(%)	
Total number	29		27		
Gender					
Male	18	62.1%	17	63.0%	1.00
Female	11	37.9%	10	37.0%	
Age					
≤18 months old	7	35%	13	48.2%	0.09
>18 months old	22	65%	14	51.8%	
Histological subtype					
Stroma-rich	5	17.2%	11	40.8%	0.07
Stroma-poor	24	82.8%	16	59.2%	
Histology					
Favorable	4	13.8%	11	40.8%	0.03
Unfavorable	25	86.2%	16	59.2%	
Stage					
Low	5	17.2%	14	51.9%	0.01
High	24	82.8%	13	48.1%	
Abdomen					
Yes	24	82.7%	19	70.3%	0.34
No	5	17.3%	8	29.7%	
Status					
Surviving	11	38.0%	25	92.7%	0.000018
Deceased	18	62.0%	2	7.3%	

\*VEGF score >2

\*\*Tumor vascular volume fraction ≤5%



**Fig. 6.** Kaplan-Meier overall survival curves for advanced stage patients (stage 3 and 4). a) The presence of high VEGF expression (score >2) and low tumor vascularity (TVVF ≤5%) were evaluated as a complex variable (red line), and these patients were associated with significantly shorter survival periods (p=0.0019) than all others (blue line); b) Patients that received (red line) or did not receive (blue line) HSCT were evaluated, and HSCT did not significantly improve patient survival (p=0.11).

In order to determine independent predictors of overall survival, multivariate analysis was performed. The full Cox proportional-hazards regression model (Stepwise method) was statistically significant (p<0.001), indicating that this model was able to distinguish between survival and non-survival. As shown in Table 5, two pre-

**TABLE 5**  
COX PROPORTIONAL-HAZARDS REGRESSION MODEL (STEPWISE METHOD)\* FOR NEUROBLASTOMA PATIENTS OVERALL SURVIVAL

Covariate	p	OR**	95% CI***of OR
High stage	0.0394	8.4436	1.1211 to 63.5956
VEGF & tumor vascularity (complex variable)****	0.0041	8.6741	1.9961 to 37.6941

\*Overall model fit  $\chi^2=28.4461$  p<0.0001

Abbreviations: \*\*Odds ratio; \*\*\* Confidence interval

\*\*\*\* VEGF expression score >2 and VVF ≤5.0%

Variables not included in the model: age, localisation, histology, transplantation



dicator variables significantly affected the model, high disease stage ( $p=0.0394$ ,  $OR=8.4436$ ,  $95\%CI$  1.1211 to 63.5956), and high VEGF expression with low tumor vascularity as a complex variable ( $p=0.0041$ ,  $OR$  8.6741,  $95\%CI$  1.9961 to 37,6941).

## Discussion

In this study low tumor vascularity in high VEGF expression tumors were associated with a lower OSR. To our knowledge, this is the first clinical study to evaluate the impact of differences in vascularity using image analysis to determine the prognosis of high VEGF expression in neuroblastoma patients. Based on a search of the literature currently available, our study represents the first analysis of vascular volume fraction as a measure of tumor angiogenesis in neuroblastoma patients. Canete et al. (2000) used a similar method, however, they examined other vascular variables<sup>5</sup>. For differences in TVVF values for surviving *vs.* deceased patients estimated using the Mann-Witney test, near statistical significance was observed. Furthermore, the TVVF values associated with the group of deceased patients were lower than the TVVF values attributed to the surviving patients. Low tumor vascularity (TVVF  $\leq 5\%$ ) was also significantly associated with a bad prognosis, and characterized patients having a shorter survival. Although this result was unpredictable, it may be due to the high ratio of patients with unfavorable biologic and clinical features that were included in this study. We hypothesize that tumors with high VEGF expression along with low levels of vascularity undergo an up-regulation of glycolysis and acid resistance, which results in a powerful growth advantage to promote unconstrained proliferation and invasion. In contrast, high vascularity (TVVF  $>5\%$ ) detected in the neuroblastoma cases examined was associated with a good prognosis, and appeared to indicate that these tumors were well-oxygenated. Although measurements and markers of angiogenesis cannot be used as surrogate measures of tumor hypoxia, we hypothesize that patients with high tumor vascularity experience a sufficient degree of tumor oxygenation and efficiency of tumor vascularization for blood delivery, which would have a significant impact on the success of treatment. Our results are contrary to those of other studies where a positive correlation between tumor angiogenesis (microvascular density) and unfavorable outcome were observed for neuroblastoma patients. For example, Meitar et al. (1996) found that a high tumor vascular index value was an independent, adverse prognostic factor in neuroblastoma<sup>3</sup>. In contrast, vascular parameters were not predictive of survival for neuroblastoma patients in a study by Canete et al. (2000) and Noguera et al. (2009)<sup>5,6</sup>. Izycka-Swieszewska et al. (2007) found that lower vascular density and pathologic-type angiogenesis were associated with a shorter survival for neuroblastoma patients<sup>7</sup>. The differences in these results could be attributed to the different methods of measurement used, differences in the evaluation of biological readouts, and differences in the ratios of pa-

tients with favorable *vs.* unfavorable biological and clinical features.

In this study tumors with high levels of VEGF expression were initially predicted to have greater tumor vascularity compared with tumors expressing low levels of VEGF. However, the difference between these two groups was not significant. Tumor vascularity also had no detectable impact on the survival of patients with low levels of VEGF expression, since all of these patients survived. In contrast, deceased patients with high levels of VEGF expression had significantly lower TVVF values compared to surviving neuroblastoma patients with high levels of VEGF expression. This result indicates that it is important to discriminate the angiogenesis associated with low *vs.* high VEGF expression, as well as the analysis for each. Accordingly, a difference in the correlation between TVVF values and VEGF expression that was evaluated using the rank sum test was identified for surviving *vs.* deceased patients. For the surviving patients, a significant positive correlation between TVVF and VEGF scores was observed, which was not observed for the deceased patients. Based on these results, we would then ask, why do deceased patients, as opposed to surviving patients, do not have TVVF values that positively correlate with VEGF expression?

It may be possible that the angiogenesis in these neuroblastomas was not affected by the actions of VEGF due to the influence of other factors that were activated during hypoxia. Pathways induced by hypoxia, particularly the hypoxia-inducible factor-1 (HIF-1) pathway, have been shown to regulate the expression of a diverse group of genes that promote tumor growth, and are involved in tissue invasion, angiogenesis, cell proliferation, glycolysis, and pH regulation<sup>41–45</sup>. Other reason for this discrepancy is that in aggressive high risk neuroblastoma, endothelial microvessels can originate from tumor cells and have a high probability of being CD 34 negative. This could be an explanation of low TVVF (based on CD34 staining) in highly VEGF expressing tumors in deceased patients. Therefore in future studies, it would be important to label and analyze tumor vascular progenitor cells. Some research indicates that they could possess resistance to chemotherapy<sup>46</sup>.

Other studies have also found that angiogenesis correlates with N-*myc* amplification, as well as poor outcome, in patients with neuroblastoma. Correspondingly, it has been hypothesized that N-*myc* may function in part by promoting angiogenesis via VEGF<sup>3</sup>. Kang J et al. (2008) pointed out the key role of PI3K/Akt/VEGF pathway for angiogenesis in neuroblastoma and the importance of N-*myc* amplification in its regulation<sup>47</sup>.

Unfortunately, however, the N-*myc* status in this study was unable to be determined for some patients due to our inability to obtain DNA of sufficient quality for analysis. However, we anticipate that this analysis will be completed on these samples in a future study.

Tumor lymphangiogenesis also affects the aggressive behavior of malignant cells. Becker L et al. (2010) found that together, upregulation of hemangiogenesis and lym-



phangiogenesis activators (VEGF-A and VEGF-D, respectively) and downregulation of hemangiogenesis and lymphangiogenesis inhibitors (VEGFR-1 and soluble form of VEGFR-2, respectively) may represent cooperative mechanisms during neuroblastoma progression<sup>48</sup>. Therefore analysis of lymphangiogenesis activators and inhibitors in patients with high VEGF expression and low TVVF could provide valuable insight.

Based on the results of this study, we would like to emphasize the significant prognostic value associated with an analysis of tumor vascularity in the context of VEGF expression and its correlation with clinicopathological characteristics of high-risk neuroblastoma patients. The multivariate analysis of survival confirmed the prognostic significance of VEGF expression along with tumor vascularity as a complex variable which was determined as an independent predictor of overall survival.

For patients whose tumors exhibited high VEGF expression together with low vascularity (based on CD34 staining), these patients were more frequently associated with advanced stage disease, and an unfavorable histology, compared to all other patients.

When only advanced tumors were analyzed, with high levels of VEGF expression and low tumor vascularity evaluated as a complex variable, patient survival could be predicted. In addition, the OSR for this group of patients was significantly lower, thereby identifying high levels of VEGF expression in combination with TVVF

values  $\leq 5\%$  to be a prognostic marker independent of clinical stage according to INSS. Furthermore, the survival of these high-risk patients was not significantly improved with HSCT. These results suggest that this subset of patients are candidates for new therapies, and the biological characteristics and mechanisms of resistance of this tumor subtype need to be further investigated.

## Conclusion

Analysis of tumor vascularity in relation to VEGF expression appears to be a good prognostic marker for high-risk neuroblastoma patients, even as a factor independent of clinical staging. When survival curves for neuroblastoma patients with high VEGF expression and low tumor vascularity were calculated with these factors treated as a complex variable, OSRs were found to be reduced. In addition, the outcome for these patients was not significantly improved by HSCT. Therefore, these patients may represent ideal candidates for receiving novel therapies for the treatment of neuroblastoma.

## Acknowledgements

We wish to thank Ivan Sunara, MD for help with data collection, and Milan Milosevic, MD for his advice in statistical analysis.

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## ODNOS IZMEĐU TUMORSKE VASKULARNOSTI I VASKULARNOG ENDOTELNOG ČIMBENIKA RASTA KAO PROGNOСТИČKIH ČIMBENIKA KOD BOLESNIKA S NEUROBLASTOMOM

### SAŽETAK

Iako je utjecaj angiogeneze na tumorsku progresiju i liječenje dosta istražen, uloga angiogeneze i njeno prognostičko značenje kod neuroblastoma u kliničkoj praksi još je nejasno. Cilj ovog rada bio je istražiti da li kod neuroblastoma tumorska vaskularnost u kombinaciji s ekspresijom vaskularnog endotelnog čimbenika rasta (VEGF) predstavlja prognostički čimbenik rizika. Pomoću anti-CD34 i anti-VEGF antitijela učinjena je imunohistokemijska analiza tumorskog tkiva pohranjenog u obliku parafinskih kocki kod 56 bolesnika s neuroblastomom. Tumorska vaskularnost procijenjena je izračunavanjem tumorske vaskularne volumne frakcije (TVVF), a ekspresija VEGF-a semikvantitativnim bodovanjem. Učinjena je odgovarajuća statistička obrada uključujući i multivarijantnu analizu preživljenja. Tumorska vaskularnost utjecala je na preživljenje bolesnika s visokom VEGF ekspresijom. Visoka VEGF ekspresija u kombinaciji s TVVF  $\leq 5\%$  predstavljala je nezavisan prognostički čimbenik rizika (Coxova regresijska analiza, p-vrijednost=0,0041; omjer mogućnosti i 95% interval pouzdanosti =8,67 (1,99–37,69). Po prvi put otkrivena je skupina visoko rizičnih bolesnika čiji su tumori imali istovremeno visoku VEGF ekspresiju i malu vaskularnost. Kod tih bolesnika zapažen je nizak postotak preživljenja (37% naspram 92,5%) ( $p < 0,0001$ ). Oni nisu imali značajno poboljšanje preživljenja niti nakon transplantacije krvotvornih matičnih stanica, te bi takvi pacijenti mogli biti kandidati za primjenu novih metoda liječenja. Navedeni rezultati upućuju na važnost međusobnog odnosa tumorske vaskularnosti i VEGF-a, jer daje bolji uvid u prognozu bolesnika s neuroblastomom.