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## Vascular endothelial growth factor in children with neuroblastoma: a retrospective analysis

Gordana Jakovljević\*<sup>1</sup>, Srana Čulić<sup>2</sup>, Jasminka Stepan<sup>1</sup>, Aleksandra Bonevski<sup>1</sup> and Sven Seiwerth<sup>3</sup>

Address: <sup>1</sup>Department of Hematology and Oncology, Pediatric Clinic, Children's Clinical Hospital Zagreb, Zagreb, Croatia, <sup>2</sup>Department of Pediatric Hematology, Oncology, Immunology and Medical Genetics, Pediatric, Clinic, Clinical Hospital Center Split, Medical School University of Split, Split, Croatia and <sup>3</sup>Institute of Pathology, Zagreb University School of Medicine, Zagreb, Croatia

Email: Gordana Jakovljević\* - [gordanajakovljevic@yahoo.com](mailto:gordanajakovljevic@yahoo.com); Srana Čulić - [srdjana.culic@st.htnet.hr](mailto:srdjana.culic@st.htnet.hr); Jasminka Stepan - [jasminka.stepan@zg.htnet.hr](mailto:jasminka.stepan@zg.htnet.hr); Aleksandra Bonevski - [a.bonevski@gmail.com](mailto:a.bonevski@gmail.com); Sven Seiwerth - [seiwerth@mef.hr](mailto:seiwerth@mef.hr)

\* Corresponding author

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### Abstract

**Background:** Despite aggressive therapy, advanced stage neuroblastoma patients have poor survival rates. Although angiogenesis correlates with advanced tumour stage and plays an important role in determining the tumour response to treatment in general, clinical data are still insufficient, and more clinical evaluations are needed to draw conclusions. The aim of this study was to evaluate vascular endothelial growth factor (VEGF) expression in patients with neuroblastoma, determine whether it correlates with other prognostic factors and/or therapeutic response, and to assess should VEGF be considered in a routine diagnostic workup.

**Materials and methods:** VEGF expression was determined by immunohistochemistry using anti-VEGF antibody in paraffin embedded primary tumour tissue from 56 neuroblastoma patients. Semiquantitative expression of VEGF was estimated and compared with gender, age, histology, disease stage, therapy, and survival. Statistical analyses, including multivariate analysis, were performed.

**Results:** VEGF expression correlated with disease stage and survival in neuroblastoma patients. Combination of VEGF expression and disease stage as a single prognostic value for survival (P-value = 0.0034; odds ratio (OR) (95%CI) = 26.17 (2.97-230.27) exhibited greater correlation with survival than individually. Hematopoietic stem cell transplantation significantly improved survival of the advanced stage patients with high VEGF expression.

**Conclusion:** VEGF expression should be considered in a routine diagnostic workup of children with neuroblastoma, especially in those more than 18 months old and with advanced disease stage. High VEGF expression at the time of disease diagnosis is a bad risk prognostic factor, and can be used to characterize subsets of patients with an unfavourable outcome.

## Background

Neuroblastoma (NB), a paediatric solid tumour of neural crest origin, is the most frequent extracranial solid malignancy in children. Despite intensive multimodal therapy, the prognosis of patients older than 1 year with advanced disease remains poor, with long term survival less than 40%. A consensus was reached in determining the neuroblastoma risk stratification schema considering age, stage and *N-myc* status [1]. In general, angiogenesis plays an important role in the progression and metastasis of malignant tumours [2]. In neuroblastoma, tumour vascularity is correlated with an aggressive phenotype [3,4]. Pro-angiogenic factors are differentially expressed in high-risk neuroblastoma [5,6]. Vascular endothelial growth factor (VEGF) is a specific endothelial cell mitogen that stimulates angiogenesis and plays a crucial role in tumour growth [7]. Overexpression of VEGF has been demonstrated in neuroblastoma, nephroblastoma, as well as in other cancers, such as colon, breast, brain, lung, malignant pleural mesothelioma, esophageal and gastric carcinomas [8-10]. In adult solid tumours VEGF expression has been successfully evaluated by immunohistochemistry, and has been reported to be an independent prognostic factor [11-15]. Recent studies have validated inhibition of VEGF as an effective antiangiogenic therapy in some of these cancers [16-18]. Although several preliminary studies have demonstrated that expression of angiogenic growth factors, including VEGF, correlate with a high-risk phenotype in neuroblastoma, clinical data are still insufficient to draw conclusions [5,9,19-21]. Therefore, further clinical studies, are needed to evaluate the possible significance of these factors for use in a routine clinical practice. Preclinical studies also suggest that antiangiogenic strategies may be effective in the treatment of neuroblastoma [22,23]. Whether inhibition of angiogenesis is a realistic approach for preventing dissemination of neuroblastoma, remains to be determined. In addition, phase I clinical trials (COG study) using the human anti-VEGF antibody, bevacizumab, in pediatric patients with refractory solid tumours reported promising results [24].

The aim of this study was to evaluate VEGF expression in patients with neuroblastoma and determine whether it correlates with other prognostic factors and/or therapeutic response. Also, we tried to assess should VEGF be considered in a routine diagnostic workup of children with neuroblastoma. Maybe these results could help in the planning further follow-up strategies and antiangiogenic therapy trials.

## Materials and methods

### *Patients and tumour samples*

Neuroblastoma tissue samples (n = 56) included in this study were retrieved from the archives of the Institute of Pathology Medical School University of Zagreb, Croatia.

They were obtained from patients treated at the Children's Clinical Hospital Zagreb between 1995 and 2008 at the beginning of disease (first biopsy). Clinical staging was classified according to The International Neuroblastoma Staging System (INSS) [1,25]. Histopathological grading was classified according to Shimada System and Shimada Age-based Pathologic Classification [26,27]. All the histological samples underwent a reevaluation and new grading (SS). Patients with stage 1, 2 and stage 4s disease (19 patients) were treated with surgery alone, or surgery and moderate-dose chemotherapy. Patients with stage 3 and 4 (37 patients) were treated with surgery combined with intensive, multiagent chemotherapy either with or without radiotherapy and/or metaiodobenzylguanidine (MIBG) therapy. Fourteen patients with advanced disease, and 3 patients with localized disease with *N-myc* amplification tumour received megatherapy (myeloablative chemotherapy) followed by autologous or allogeneic hematopoietic stem cell transplantation. As hematopoietic stem cell transplantation for our high-risk patients was started in 1999, there were 2 groups of high risk patients, either treated with or without stem cell transplantation (Table 1).

### *Immunohistochemistry*

Immunohistochemical analysis was performed on formalin-fixed paraffin-embedded tumour sections. Following surgery, specimens were fixed in 4% buffered formalin for at least 24 h, embedded in paraffin, and stored at room temperature until sectioning. Serial 4-5  $\mu$ m sections were cut and adhered onto microscope slides. Paraffin was removed from the sections using Xylene; the samples were rehydrated, and processed using the streptavidin-biotin-peroxidase complex immunohistochemical technique. To ascertain immunoreactivity, antigen unmasking was performed by microwave treatment with 10 mM citrate buffer. Incubation with 10% normal goat serum in phosphate-buffered saline (PBS) was performed to eliminate nonspecific staining. After incubating for five minutes in 3% hydrogen peroxide, the slides were then incubated for 30 minutes at room temperature with primary antibody, VEGF-specific mouse monoclonal IgG (dilution 1:25; Dako). Detection of primary antibody was achieved with a secondary antibody detection kit (LSAB+kit, Dako, Denmark). Bound antigens were visualized using 3, 3'-diaminobenzidine as a chromogen. Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted for analysis. Negative control was performed by incubating with Tris-buffered saline (TBS) instead of primary antibody. Colon carcinoma, shown to strongly express VEGF, was used as positive control.

### *Immunohistochemical analysis*

We intended to focus on the positivity in viable tumor tissue and to analyze the "hot spots" of immunoreactivity.

**Table 1: Patient characteristics**

Characteristics	No. patients
<b>Total number</b>	<b>56</b>
Gender	
Male	35
Female	21
Age	
Median 35.5 months	
Range 2 months - 12 years	
>18 months old	36
≤ 18 months old	20
Histologic subtype	
Stroma-rich	
Well differentiated	3
Intermixed	10
Focal nodular	3
Stroma-poor	
Undifferentiated	30
Differentiating	10
Histology	
Favourable	23
Unfavourable	33
Stage	
1	3
2	15
3	20
4	17
4s *	1
Treatment	
S	3
S/CTH	32
S/CTH/MIBGT	2
S/CTH/RT	2
S/CTH/BMT	14
S/CTH/MIBGT/BMT	2
S/CTH/BMT/RT	1
Survival	
Alive	35
Dead	21

Abbreviations: 4s \* in infants with small primary tumours and metastatic disease involving the skin, liver, limited infiltration of the bone marrow, and can spontaneously regress; S, surgery; CTH, chemotherapy; MIBGT, metaiodobenzylguanidine therapy; BMT, bone marrow transplant; RT, radiotherapy

The cells showing positive staining for VEGF were defined morphologically by hematoxylin and eosin (H&E) staining, using the serial sections. We compared immunohistochemical stains with preceding H&E slides to ascertain the exact location of immunoreactivity. Only cancer cells immunostained for VEGF were measured. The number of

positive cells per 200 × field was assessed. In each slide three fields were evaluated. Semiquantitative expression levels of VEGF were determined by assessing both the percentage and intensity of stained tumour cells. The percentage of positive cells was rated as follows: cases with <1% positive cells were rated as 0 point, 1-25% positive cells were rated 1 point; 26-50% positive cells, 2 points; 51-75% positive cells, 3 points; 76-100% positive cells, 4 points. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; 3 points, strong intensity. Points for staining intensity and percentage of positive cells were added, and specimens were classified into 2 groups according to their overall score: weak expression 0-2 points; and strong expression, 3-7 points.

### Statistical analysis

Descriptive statistics and 95% confidence intervals were calculated to describe data. Data distribution was analyzed with the Smirnov-Kolmogorov test. According to the type of distribution, an appropriate parametric or an equivalent non-parametric test was used. The cutoff value for determining VEGF low and high expression score was performed by the receiver operating characteristic (ROC) curve analysis [28]. The relationship between VEGF expression data and gender, age, tumour stage, histology, stem cell transplantation therapy and survival was estimated by applying the Fisher's exact test [29]. Spearman's coefficient of rank correlation ( $\rho$ ) was determined to assess correlation between tumour stage and VEGF score, as well as VEGF score and survival. 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 [30]. Statistical significance of differences in survival between the patients groups with respect to gender, age, stage, histology, VEGF staining intensity and transplantation therapy was estimated using the log-rank test. Statistical analysis was performed using GrafPad Prism 5 (GrafPad Software, Inc, San Diego, CA) computer program. The Cox proportional hazards model was used for multivariate analysis to determine independent predictors of overall survival, and was carried out using MedCalc version 10.4 (MedCalc Software bvba, Mariakerke, Belgium) computer program [31]. Differences were considered significant at  $P < 0.05$ .

## Results

### Patient sample classifications

We examined tumour samples of 56 NB patients at disease onset. Patient characteristics are detailed in Table 1 and Table 2. The median patient follow-up time was 27 months (range, 1.0 to 180.0 months). The overall survival rate was 62,5%. Regarding age and gender at diagnosis, the mean age was 35,5 months (range 2 months to 12

**Table 2: Patient characteristics**

Patient no./sex/age (mo) at diagnosis	Tumour stage at diagnosis	Histologic subtype†	Age-linked classification‡	VEGF% points§	VEGF intensity¶	VEGF score®	Clinical outcome@
1/m/18	2	1a	U	1	2	3	S
2/m/36	2	4	U	1	1	2	S
3/m/18	2	1b	F	2	2	4	S
4/m/18	2	1a	U	2	2	4	S
5/f/37	2	2	F	1	1	2	S
6/m/10	3	1b	F	1	1	2	S
7/f/34	2	3	F	1	1	2	S
8/m/121	2	3	F	2	2	4	S
9/m/71	3	3	F	1	2	3	D*
10/m/3	1	1a	F	3	2	5	S
11/f/3	4s*	1a	U	1	1	2	S
12/f/33	2	1a	U	1	2	3	S
13/m/48	3	1a	U	4	3	7	S
14/m/53	3	2	F	1	2	3	S
15/m/18	3	1a	U	0	0	0	S
16/f/46	2	2	F	2	3	5	S
17/f/2	2	1b	F	4	3	7	S
18/m/78	2	2	F	1	1	2	S
19/m/4	3	1b	F	2	3	5	S
20/m/2	2	1a	F	1	2	3	S
21/m/2	2	1a	U	0	0	0	S
22/f/18	3	1b	F	1	2	3	S
23/f/14	2	1b	F	1	1	2	S
24/m/72	3	2	F	3	3	6	S
25/m/144	4	1a	U	2	3	5	D
26/f/24	4	1a	U	2	3	5	D
27/m/120	4	1a	U	2	3	5	D
28/m/38	3	2	F	2	2	4	D
29/m/120	4	1a	U	1	2	3	D
30/m/35	4	1a	U	1	2	3	D
31/f/61	4	1a	U	2	2	4	D
32/m/97	2	1a	U	1	2	3	D
33/m/84	3	1b	U	3	2	5	D
34/f/23	4	1b	U	2	2	4	D
35/f/42	4	1a	U	4	3	7	D
36/f/5	4	1a	U	2	3	5	D
37/m/36	3	1a	U	3	2	5	D
38/f/48	4	1a	U	4	3	7	D
39/m/10	4	1a	U	1	2	3	D
40/f/122	2	1a	U	1	1	2	S
41/m/38	4	1a	U	3	3	6	S
42/f/35	3	2	F	1	4	6	S
43/m/30	1	2	U	1	1	2	S
44/m/3	4	1b	F	2	2	4	S
45/f/12	4	1b	F	2	3	5	S
46/f/17	3	1a	U	2	3	5	S
47/m/18	4	1a	U	2	3	5	S
48/m/36	3	2	F	1	1	2	S
49/m/66	4	1b	U	1	2	3	S
50/m/12	4	1a	F	2	3	5	S
51/m/36	3	2	F	3	2	5	S
52/f/35	4	4	U	1	3	4	D
53/f/54	4	1a	U	1	2	3	D
54/m/54	4	4	U	2	3	5	D
55/m/60	3	1a	U	3	1	4	D
56/f/56	4	1b	U	1	2	3	D

**Table 2: Patient characteristics (Continued)**


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Tumour staging at diagnosis according to INSS<sup>25</sup>;  
† Shimada histopathologic classification<sup>26</sup> 1a: Stroma poor, undifferentiated; 1b: Stroma poor, differentiating; 2: Stroma rich, intermixed; 3: Stroma rich, well differentiated; 4: Stroma rich, focal nodular  
‡ Prognostic groups according to the age-linked classification of Shimada<sup>26</sup> F: favourable; U: unfavourable;  
§ VEGF % (percentage of positive VEGF cells) 0 point: <1% positive cells, 1 point: 1-25% positive cells; 2 points: 26-50% positive cells; 3 points: 51-75% positive cells; 4 points: 76-100% positive cells  
¶ VEGF intensity (staining intensity) 1: weak intensity; 2: moderate intensity; 3: strong intensity  
© VEGF score: points for staining intensity and percentage of positive cells were added  
@ S: surviving, D: dead, D\*: one patient died in relapse of tumour 5,5 yr after diagnosis  
4S\* in a infant with small primary tumour and metastatic disease involving the skin, liver, limited infiltration of the bone marrow

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years), 20 patients (35.7%) were  $\leq 18$  months of age, and 36 patients (64.3%) were  $>18$  months old. 35 patients (62.5%) were males, and 21 patients (37.5%) females. Depending of the disease stage, we separated our patients into two groups: low stage (stage 1 and 2) and high stage (stage 3 and 4), as well as favourable and unfavourable histology according to the criteria reported by Shimada, *et al* [26,27]. Thirty-seven patients had high stage disease and eighteen had low stage disease. One patient had 4S stage disease. Twenty-three patients had favourable and thirty-three patients had unfavourable histology. There was no statistically significant correlation between age ( $\leq 18$  months/ $> 18$  months) and disease stage (low/high) ( $P = 0.244$ ), or between stage and histology (favourable/unfavourable) ( $P = 0.750$ ) as determined by Fisher's exact test. Also no significant correlation between histology and age ( $\leq 18$  months/ $> 18$  months) was seen ( $P = 0.209$ ).

#### **VEGF is expressed in NB samples**

According to the criteria proposed by Volms, *et al.*, brown granules in the cytoplasm of tumour cells were identified to be VEGF positive [32]. VEGF staining was found in 54 of 56 tumours (96.4%), and only 2 tumours (3.6%) had negative VEGF immunoreactivity. Most of the specimens (76, 7%) had 1-50% positive tumour cells, and 76.8% of specimens had moderate to strong staining intensity. By assessing both the percentage and intensity of stained tumour cells, specimens were classified into 2 groups according to their overall score. By ROC curve analysis, and cut off value of  $>2.5$  (sensitivity 100%, specificity 56.25%), tumours were distinguished with high (3-7) and low (0-2) VEGF expression scores. Low VEGF expression scores were found in 12 NB (21.4%) and high expression score in 44 NB (78.6%) (Table 3). Figure 1 is showing VEGF immunohistochemical staining in different NB pathohistological sections.

#### **Correlation of VEGF expression with clinicopathological characteristics and survival**

VEGF expression and clinicopathological characteristics are detailed in Table 4. Fisher's exact test was performed. We did not observe significant correlation between VEGF expression (high/low) and gender ( $P = 0.7477$ ), age  $>18$  months/ $\leq 18$  months old ( $P = 0.2701$ ), or histology

(favourable/unfavourable) ( $P = 0.27$ ). Also, there was no significant difference in VEGF expression between the transplant and non-transplant patients ( $P = 0.7378$ ).

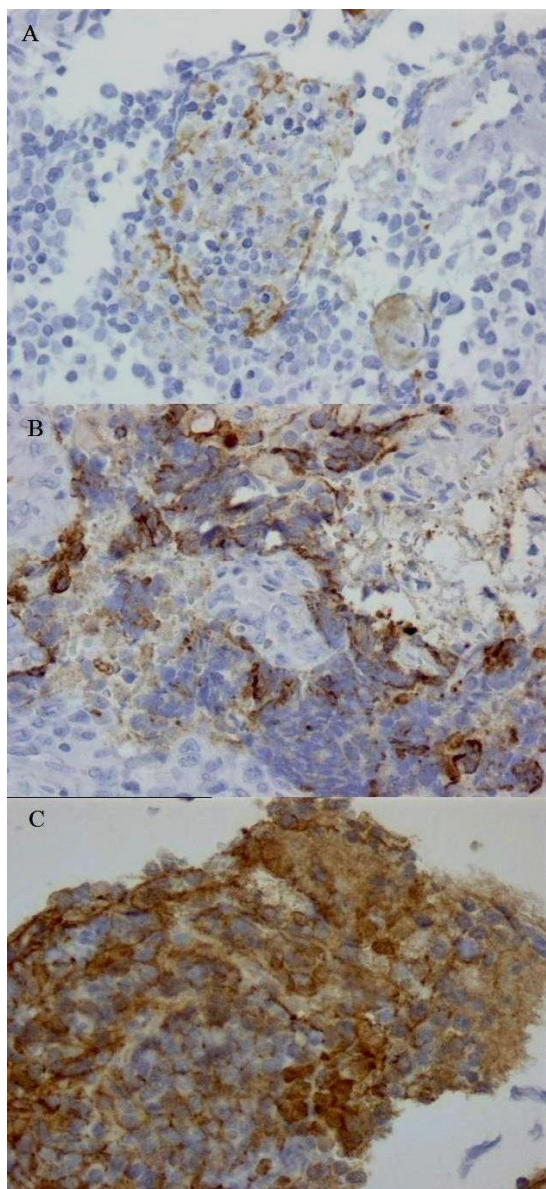
There was significant association between advanced disease stage and high VEGF expression as determined by Fisher exact test ( $P = 0.0014$ ), and significant correlation between high VEGF expression score and high tumour stage as determined by Spearman's coefficient of rank, ( $\rho = 0.453$ ,  $P = 0.0005$ ).

The VEGF expression score was significantly higher in the group of non-survival patients compared to the group of patients that survived more than 5 years, as determined by Mann Whitney test ( $P < 0.0001$ ). Also, significant correlation between VEGF expression and survival was determined by Spearman's coefficient of rank ( $\rho = -0.472$ ,  $P = 0.0002$ ). All patients with low VEGF expression score survived.

Interestingly, in the group of patients  $\leq 18$  months old we did not observe any correlation between VEGF expression and tumour stage (Spearman's coefficient of rank  $\rho = 0.17$ ,  $P = 0.46$ ), opposite to the patients  $> 18$  months old ( $\rho = 0.635$ ,  $P < 0.0001$ ). In the same group of patients ( $\leq 18$  months old), we also did not observe any correlation between VEGF expression score and survival (Spearman's coefficient of rank  $\rho = 0.19$ ,  $P = 0.42$ ; Fisher's exact test  $P = 1.0$ ), contrary to the group of patients  $> 18$  months old (Spearman's coefficient of rank  $\rho = 0.49$ ,  $P = 0.0023$ ; Fisher's exact test  $P = 0.0076$ ), (Figure 2).

#### **Univariate survival analysis**

Log-rank test was performed. There were significant differences in survival rates in the groups of patients with  $\leq$  and  $> 18$  months old ( $P = 0.0069$ ; Table 5). Patients  $> 18$  months old had lower survival rate than patients  $\leq 18$  months old. Patients with advanced stage tumours (Stage 3, 4), had lower survival rate when compared to patients with low stage tumours ( $P = 0.0006$ ; Table 5). There were significant differences in survival rates in the groups of patients with favourable and unfavourable histology ( $P < 0.0001$ ; Table 5). Patients with high VEGF expression had short median OS (30 months). Survival curve of the VEGF



**Figure 1**  
**Immunohistochemical staining for VEGF in different NB pathohistological sections.** Low VEGF expression score with low grade intensity and 1-25% tumour cell positivity (**A**); High VEGF expression score with high grade intensity and 25-50% tumour cell positivity (**B**); High VEGF expression score with moderate grade intensity and 75-100% tumour cell positivity (**C**). Objective = 40x.

low expression group was significantly higher, and OS longer, compared to the VEGF high expression group ( $P = 0.0053$ ; Figure 3, Table 5). Survival was not correlated with sex ( $P = 0.45$ ; Table 5).

**Table 3: Immunoreactivity of VEGF, and the number of patients**

Category	Number of patients (%)	alive/dead
Percentage of positive tumour cells (P)		
<1%	2 (3.6%)	2/0
1-25%	25 (44.6%)	17/8
26-50%	18 (32.1%)	10/8
51-75%	7 (12.5%)	4/3
76-100%	4 (7.1%)	2/2
Staining intensity (I)		
Negative	2 (3.6%)	2/0
Weak	11 (19.6%)	10/1
Moderate	24 (42.9%)	12/12
Strong	19 (33.9%)	11/8
Expression score (P+I)		
Low (0-2)	12 (21.4%)	12/0
High (3-7)	44 (78.6%)	23/21

**High risk patients**

Patients with high disease stage (Stage 3, 4) and high VEGF expression score (score 3-7) had short median OS (24 months). These patients had significantly lower survival rate than all other patients ( $p < 0.0001$ ; Table 5, Figure 4). The non-transplant patients with high stage disease and high VEGF expression score (high risk patients), had the shortest median OS (13 months) and significantly lower survival rate when compared to all other (low risk) non transplant patients ( $p < 0.0001$ ). Among the high-risk patients (high stage and high VEGF expression), those patients who had bone marrow transplants had significantly better survival rate (undefined median OS) when compared to non-transplant patients (median OS 13 months) ( $p = 0.0237$ ).

**Tumour stage and VEGF expression, as one combined variable - the significant mortality predictor by multivariate analysis**

The full Cox proportional-hazards regression model containing all predictors was statistically significant ( $P < 0.001$ ), indicating that this model was able to distinguish between survival and non-survival. As shown in Table 6, three predictor variables significantly affected the model, unfavourable histology, high disease stage, and transplantation. Although we did not demonstrate the role of VEGF as an independent prognostic factor by multivariate analysis, the combination of high tumour stage and high VEGF expression as one complex predictor variable, became the strongest mortality predictor by Cox proportional-hazards regression model (OR = 26.1695, 95% CI = 2.9741 to 230.2670,  $P = 0.0034$ ; Table 7). These results showed that prognostic prediction might be improved by taking into account both VEGF expression and disease stage.

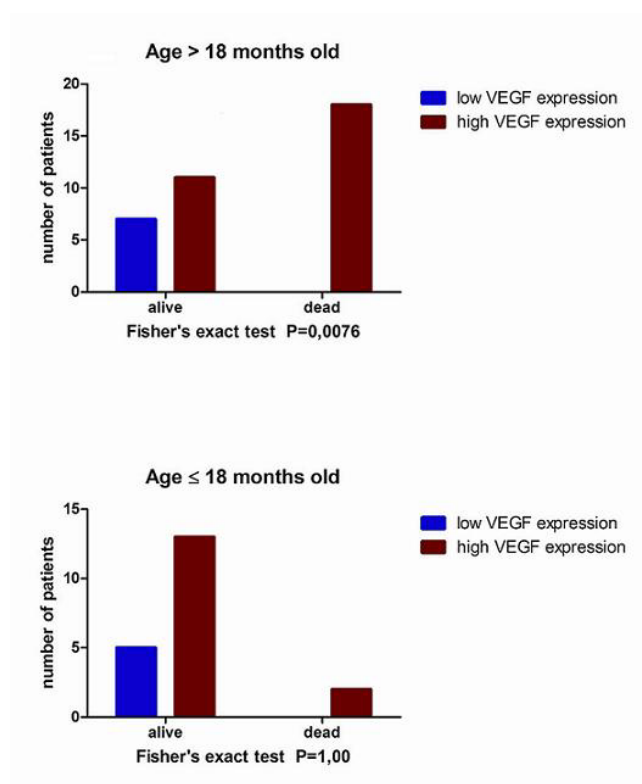


**Table 4: VEGF expression and other clinicopathologic factors**

Characteristics	VEGF score	
	Low	High
	No. patients	
Total number	12	44
Gender		
Male	7	28
Female	5	16
Age		
>18 months old	4	32
≤ 18 months old	8	12
Histologic subtype		
Stroma-rich		
Well differentiated	1	2
Intermixed	3	7
Focal nodular	1	2
Stroma-poor		
Undifferentiated	6	24
Differentiating	1	9
Histology		
Favourable	5	18
Unfavourable	7	26
Stage		
1	1	2
2	7	8
3	3	17
4	0	17
4s	1	0
Transplant		
No	9	30
Yes	3	14
Survival		
Alive	12	23
Dead	0	21

**Discussion**

So far, in some adult solid tumours semi-quantitative VEGF expression has been successfully evaluated by immunohistochemistry, and VEGF has been reported to be an independent prognostic factor [11-15]. We performed similar investigation in the cohort of patients with neuroblastoma which is the most frequent extra cranial solid malignancy in children and has a great mortality rate. In order to evaluate the prognostic significance of VEGF expression in NB patients, and estimate its diagnostic usefulness in a routine clinical practice, we have attempted to establish semi-quantitative VEGF score. As we intended to focus on positivity in viable tumour tissue, the most reliable method was immunohistochemistry. To our knowledge, this is the first time that VEGF immuno-



**Figure 2**  
**The impact of VEGF on survival in different age groups.** Expression of VEGF has impact on survival in the patients > 18 months old (A). VEGF expression is not statistically significant for survival in the group of patients ≤ 18 months old (B).

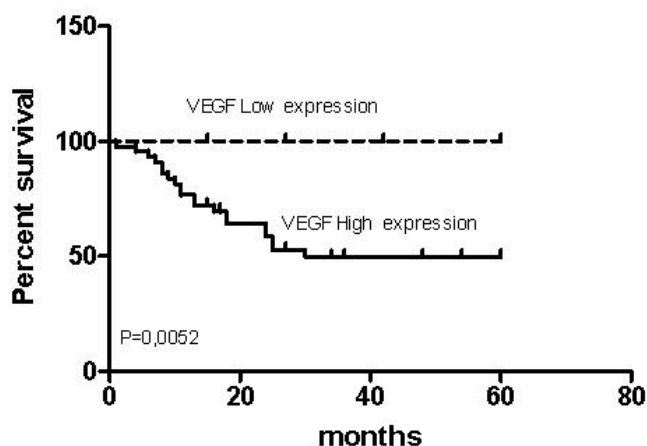
histochemistry score has been evaluated in NB patients. We analyzed "hot spots" of immunoreactivity which could be easily missed by other techniques.

In our cohort VEGF positive immunostaining was found in 96.4% of all NB tumour specimens tested, with most samples having moderate to strong staining intensity (78.6%). Despite some differences in scoring systems described in different studies, the frequency of VEGF positive tumours in this study was higher than in adult cancers [11,13-15]. It can be explained by NB-specific biology and significant tumour tissue hypoxia [8,33,34].

No correlation between VEGF expression and gender, age, or histology was found. However, there was significant correlation between high stage and high VEGF expression, and between high VEGF expression and short survival. Contrary to the patients with high VEGF expression, all patients with low VEGF expression survived.

These results support the hypothesis of a dual function for VEGF in autocrine tumour growth. In addition to its





**Figure 3**  
**VEGF and survival by Kaplan-Meier analysis.** Expression of VEGF is a significant prognostic factor. Kaplan-Meier analysis of overall survival for all NB patients according to high and low VEGF expression (P = 0.0052).

effects on angiogenesis, VEGF may affect NB cell growth, directly, and could be an autocrine growth factor [35]. In addition to stimulating angiogenesis in tumour growth, VEGF also mediates neuroprotection promoting neuroblastoma cellular survival by increasing Bcl-2 and pro-caspase 3 expressions [36].

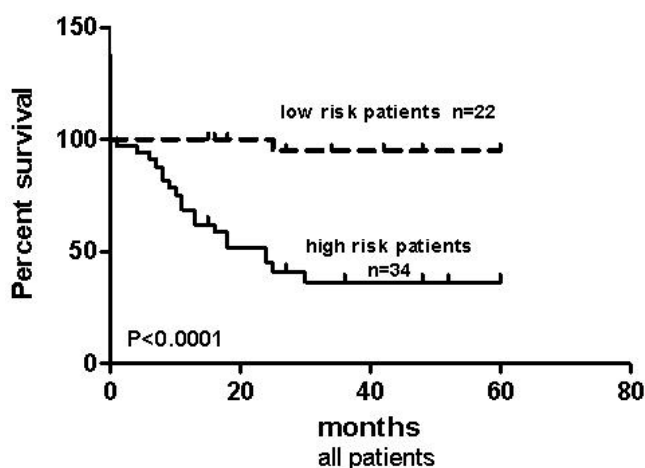
Additional trials also confirm the correlation between VEGF expression and the grade of NB [5,35,37,38]. VEGF levels in the sera of metastatic NB patients and other paediatric solid tumour patients are much higher than in non-metastatic patients [39]. Other authors did not find correlation between VEGF expression and disease stage, but they found association between high VEGF expression and unfavourable histology [19]. Perhaps, the differences between the results were caused by small patient groups and different methods of VEGF evaluation. Larger multicentric studies are needed to obtain more reproducible results. Also, new experimental models to study the angiogenic and invasive potential of NB tumours cells are still needed in order to further investigate human tumour progression and anti-angiogenic molecule screening [40,41].

As we mentioned, we found significant correlation between high stage and high VEGF expression, and strong correlation between high VEGF expression and short survival in the cohort of our NB patient, except in the patients with age ≤ 18 months old. Patients younger than 18 months have a good prognosis, and spontaneous tumour maturation/regression can happen due to favourable autocrine and paracrine interactions among tumour cells. We suppose that in these tumours the effects of VEGF could be diminished by stimulators of tumour maturation, but further prospective designed neuroblastoma angiogenesis/anti-angiogenesis studies are needed to

**Table 5: Overall survival rates and univariate analysis of patients with NB according to clinicopathologic factors**

Variable	Number of patients	Overall survival rates	Log-rank Test
Gender			
boys	35	68.6%	P = 0.4497
girls	21	57.1%	
Age			
≤ 18 months	20	90%	P = 0.0069
> 18 months	36	50%	
Stage			
high	37	50.0%	P = 0.0006
low	18	94.4%	
Histology			
favourable	23	95.7%	P < 0.0001
unfavourable	33	42.4%	
VEGF expression			
high	44	54.5%	P = 0.0053
low	12	100.0%	
Risk group			
high*	34	44.1%	P < 0.0001
low**	22	95.5%	

Abbreviations: \*high VEGF expression (score 3-7) together with high disease stage (Stage III, IV); \*\*all others



**Figure 4 High risk group and survival by Kaplan-Meier analysis.** High risk group has short overall survival (OS) (24.00 months). Kaplan-Meier analysis of OS according to risk groups\* for NB patients ( $P < 0.0001$ ). Abbreviation: risk groups\* = high risk group: patients with high disease stage (stage III, IV) and high VEGF expression score (3-7); low risk group: all other patients.

draw conclusions. Maybe one of these factors is Pigment epithelium-derived factor (PEDF) which is inhibitor of angiogenesis and inducer of neural differentiation [42]. In the study of Crawford SE, *et al.* (2001), "undifferentiated neuroblastoma tumour cell secretions were angiogenic primarily due to vascular endothelial growth factor, and secretions of Schwann cells were anti-angiogenic due to PEDF. In addition, PEDF was the major factor responsible for Schwann cell's ability to induce tumour cell differentiation in vitro and recombinant PEDF had the same effect in vitro and in vivo. Thus PEDF may serve as a multifunctional antitumor agent in neuroblastomas" [42].

Survival rates of our NB patients were analyzed according to gender, age, stage, histology, and VEGF expression. In accordance with previous reports (1), age > 18 months was a significant prognostic factor. By univariate analysis,

**Table 6: Cox proportional-hazards regression model\* for NB patients overall survival**

Covariate	P	OR**	95% CI***of OR
High stage	0.0238	11.3891	1.3949 to 92.9926
VEGF expression score	0.3831	1.1790	0.8159 to 1.7038
Unfavourable histology	0.0073	16.4610	2.1432 to 126.4302
Age older than 18 months	0.1988	3.0418	0.5624 to 16.4532
Without transplantation	0.0295	3.2280	1.1298 to 9.2227

\*Overall model fit  $\chi^2 = 42.105$   $P < 0.0001$

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

tumour stage, favourable/unfavourable histology and VEGF immunoreactivity were also found to be significant prognostic factors for overall survival. By combining VEGF expression and disease stage the prognostic value for survival was even more improved. Patients with high tumour stage and high VEGF expression were high-risk, with short median of overall survival (OS) (24 months). Among this group, there were significant differences in OS between transplant (undefined median OS), and non-transplant patients (13 months median OS). Multimodal therapy with hematopoietic stem cell transplantation significantly improved survival of these high risk patients. Perhaps survival rates could be further improved by adding bevacizumab in their therapy because in addition to its antiangiogenic and proapoptotic properties, bevacizumab can transiently "normalize" the abnormal structure and function of tumour vasculature to make it more efficient for oxygen and drug delivery [43]. If bevacizumab treatment suppresses NB progression in the setting of minimal residual disease, it would likely be a good therapy option post stem cell transplantation for high VEGF expression, high risk patients [44].

In multivariate analysis by the Cox regression model, Shimada histopathology age-linked classification, tumour stage and hematopoietic stem cell transplantation had significance as independent prognostic factors for overall survival. Although we did not demonstrate the role of VEGF expression score as an independent prognostic factor by multivariate analysis, the combination of high tumour stage and high VEGF expression as one complex predictor variable was the strongest mortality predictor by Cox proportional-hazards regression model.

As tumour angiogenesis correlates with metastatic disease, *N-myc* amplification, and poor outcome in human neuroblastoma, and some studies suggest that *N-myc* may function in part by promoting angiogenesis via VEGF, it would be important to compare *N-myc* amplification with VEGF expression in the clinical trials [3,41]. Due to our failure to obtain DNA of sufficient quality when we tried to prepare paraffin-embedded material for molecular biology study, we were not able to correlate *N-myc* amplification level and VEGF expression. Nevertheless, our results indicated that VEGF expression should be considered in the diagnostic workup of children with neuroblastoma, especially in those older than 18 months and with advanced disease stage.

**Conclusion**

This study suggests that VEGF, a critical regulator of tumour angiogenesis, might serve as an important neuroblastoma prognostic biological marker in a routine clinical practice. It can be used to identify neuroblastoma high risk patients in combination with tumour stage and

**Table 7: Cox proportional-hazards regression model\* including High risk\*\* covariate for NB patients overall survival**

Covariate	P	OR***	95%CI****of OR
High risk	0.0034	26.1695	2.9741 to 230.2670
Without transplantation	0.0111	4.2160	1.3949 to 12.7425
Unfavourable histology	0.0052	20.4384	2.4824 to 168.2770
Age older than 18 months	0.6819	1.4019	0.2809 to 6.9955

\*Overall model fit  $\chi^2 = 45.904$  P < 0.0001

Abbreviations: \*\* High VEGF expression (score3-7) together with high disease stage (Stage III, IV);\*\*\*Odds ratio; \*\*\*\*Confidence interval

other relevant risk factors. Furthermore, VEGF expression would be useful in determining the necessity for stem cell transplantation, determining follow-up strategies and anti-angiogenic therapy trials.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

GJ made conception, designed and coordinated the study, collected samples, analyzed data, carried out data interpretation, and drafted the manuscript. SS participated in the conception and design of the study, performed the reevaluation and new grading of the histological samples, carried out the immunohistological analysis, and participated in drafting of manuscript. SC participated in the conception and design of study, and in drafting of manuscript. JS and AB helped to collect the samples and to draft the manuscript. All authors read and approved the final manuscript.

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