A correlation of microvascular density and proliferative activity to clinical and histological characteristics in neuroblastoma*

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Seventy-four neuroblastoma patients were analyzed according to the clinical data including age, stage, bone metastases, primary tumor localization, tumor diameter, LDH, and serum ferritin. Histological examination of tumor specimens comprised calculation of proliferative index (PI) on slides stained with anti Ki-67 antibody and assessment of microvascular density (MVD) on anti-CD34 stained sections. Wide range of PI (1.5–79; median 37.8%) and MVD (41–385; median 172/mm^2) values was observed. Significant relationship between higher PI and tumor diameter more than 5 cm (40.3 vs 37.2%) was found. Lower PI was found more frequently in stroma-rich tumors. Significantly higher median MVD was found in infant tumors and in smaller tumors <5 cm. Tendency to inverse relationship between PI and MVD was observed. The high values of both PI and MVD were found in some aggressive tumors in patients >1-year old. We evaluated the new parameter: proliferative-vascular index (PVI) as PVI=PIxMVD which ranged from 213–18333. Among eleven patients >1 year old, with PVI >7000, seven (64%) had a poor outcome within the mean period of 22 months. Our results suggest that the simultaneous estimation of proliferative activity and vascularity of neuroblastomas could be studied as a prognostic indicator. Further investigations are needed to confirm this finding.

Key words: proliferative index, angiogenesis, microvascular density, neuroblastoma

Neuroblastoma (NB), the most frequent extracranial solid tumor in children is characterized by its biological heterogeneity during the clinical course [1]. Up until now multiple prognostic factors were established to predict the aggressiveness of the tumor in individual cases and to classify the patient into the proper risk-group [1, 2]. The most commonly used classification, dividing the patients into three risk groups is based on clinical factors like stage, age at diagnosis, and the presence of characteristic genetic feature-MYCN amplification [1]. Despite this practical system, some patients still have an unpredictable course of disease. This stimulates the need for further research to determine other prognostic factors in neuroblastic tumors.

The basic feature of cancer cells is the potential for uncontrolled proliferation. For this reason the cancer is sometimes called the disease of the cell cycle. Studies performed on different malignancies showed that there is a relationship between proliferative activity and prognosis of the tumor [3–5]. In neuroblastoma, the mitosis-karyorrhexis index, which was proposed by SHIMADA and accepted as an International Pathological Classification (INPC), is one of the important indicators of favorable/ unfavorable pathological classification [6, 7]. The proliferation index (PI) assessed with immunohistochemical staining against Ki-67 or PCNA provides a more precise view of proliferation [8]. Studies on PI of NB tumors in children above one year of age, documented the significant correlation of Ki-67 expression with unfavourable histology, advanced clinical stages, and unfavourable genetic prognostic markers such as MYCN amplification and aneuploidy [9–12].

Proliferating cells demand oxygen and nutritive supply, so one of the crucial processes in tumor progression and its metastatic ability is angiogenesis [13–15]. The most widely used method to evaluate angiogenesis intensity in neoplastic tissue is the assessment of microvascular density (MVD). This pa-

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rameter is usually measured in "hot spots" representative tissue slides of the tumor with the highest number of vessels. To visualize intratumoral vessels, immunohistochemical techniques are widely used and von Willebrand factor (vWf), CD31 or CD34 are the most frequently used antibodies [16, 17]. In some tumors MVD was proved as reliable prognostic information [14, 16, 18, 19]. Recent studies on angiogenesis in neuroblastoma revealed importance for growth characteristics of these tumors [20, 21]. "Switch on" angiogenesis particulary in advanced NB tumors was also confirmed in several studies [10, 21, 22].

The purpose of this study was to investigate the association of quantitatively assesed microvascular density and the cell proliferation index with clinical and morphological characteristics of NB.

Patients and methods

Patients. Seventy-four neuroblastoma patients treated in 7 pediatric oncological centres in Poland between 1994 and 2004 were analyzed. The patients clinical characteristics analyzed included age, stage, presence of bone metastases, localisation, maximal diameter of the primary tumor and the biochemical data (plasma LDH and ferritin) at diagnosis as well as the treatment response.

Pathology. The histopathological analysis was performed on formalin fixed paraffin embedded tissue specimens from primary tumors. The diagnosis was assessed on HE stained slides based on INPC criteria.

Immunohistochemistry. Five μm serial sections were cut from representative blocks for immunohistochemical staining. Monoclonal antibodies against CD34 (Mouse Anti-Human CD34 Class I Clone BL-3C5) and Ki-67 (Mouse Anti-Human Ki-67 Antigen Clone MIB-1) produced by DAKO (Glostrup, Denmark) were used in all the cases. In 20 randomly selected cases antibodies against vWf (Rabbit Anti-Human Primary Factor VIII Related Antigen, DAKO, Glosrup, Denmark) were used additionally to test the quantitative results with another antibody. The slides were incubated with the appropriate primary antibody at 4 °C for 20 hours. The designation of antigens was performed using the panel of biotinylated secondary antibodies and streptavidin conjugated with peroxidase (method LSAB2-DAKO).

Diaminobenzidine (DAB) was used as a chromogen. Appropriate positive and negative controls were performed.

The number of microvessels highlighted with CD34 and vWf did not differ in 20 tested cases (p=0.79). The quality of reactions was better in CD34 staining. Because of that only results of CD34 stained slides were chosen for further analyses.

The quantitative study was carried out using the image semi-automatically analyzer system (program Multiscan v.5.10 (CSS), camera Bischke CCD-FS-2012P, microscope Olympus BX 50).

Microvascular density (MVD). The tumor fields with the highest number of the blood vessels visualized with CD34 were identified at 40x and 100x magnification. The vessels were manually pointed and automatically counted on computer images at 200x magnification; each field area was 0.643 mm^2. Each group of endothelial cells immunopositive for CD34 separated from the surrounding vessels, stroma and neoplastic cells were counted as a separate blood vessel. The mean value of the three measurements, counted in 1 mm^2, was used as the vascular density.

Proliferation activity (PI). The tumor fields with the highest number of Ki-67 positive cells (hot spots) were identified at 40x and 200x magnification. The positive nuclei in a total of at least 1000 nuclei were counted at 400x magnification. The above percentage value was identified as the proliferative index (PI).

Statistical analysis. Statistical analysis was performed using the Kruskall-Wallis test and the Mann-Whitney U test for non-normally distributed variables. A p value <0.05 was considered significant.

Results

Clinical analyses. The patient's clinical data are summarized in Table 1. Most patients presented were of unfavorable clinical prognostic factors, e.g. age >1 year old (61%) and advanced disease – III or IV clinical stage (64%). Primary tumors were most often (64%) localized in the adrenal glands. Primary tumors, greater than 5 cm, were found in 63% of the patients. Unfavorable clinical features were associated with biochemical markers of poor prognosis, such as LDH and ferritin plasma levels. Follow up ranged from 3 months to 10 years (mean 2 years). The therapy regimens were based mainly on conventional multidrug chemotherapy containing the cytostatics as follows: anthracycline, vincristin, etopozid, teniposide, dacarbazine, cyclophosphamide, ifosfamide, cisplatin, cycloplatin [23, 24]. In 17 cases radiotherapy was used after and at the end of chemotherapy. Myeloablative therapy, according to HR-NBL-1 SIOP protocol, was used in 7 patients with disseminated disease. Surgery with total or partial tumor resection was performed on 48 (65%) patients, and inoperable tumors were found in 26 (35%) patients. Complete remission was achieved in 52 (68%) patients, and poor outcome (death of progresion) was noted in 23 (31%) patients. Four of those were infants with unoperable tumors of stage IV and they achieved complete remission with chemotherapy treatment alone. Complete remission was achieved in 50 (68%) patients and poor outcome (death of progression) was noted in 23 (31%) patients.

In morphological analysis there were 59 NB Schwannian stroma-poor and 15 NB Schwannian stroma-rich cases. Half of all 74 tumor specimens were analyzed before oncological treatment and were classified as the untreated group, the rest were analyzed after induction chemotherapy and were classified as pretreated group. Among the untreated group 34/37 (92%) belonged to "stroma-poor" tumor types, including

3 undifferentiated, 22 poorly differentiated, and 9 differentiating neuroblastoma types and 3/37 were diagnosed as ganglioneuroblastoma intermixed type. In the pretreated group there were 12 ganglioneuroblastomas (12/37=32%) and 25 Schwannian stroma-poor tumors.

Proliferation index (PI). In all 74 analyzed immunostained tumor sections PI ranged from 1.5% to 79.6% with a mean of 33.5%, and a median of 35.1%. Significant differences (p=0.001) of median PI value were revealed between Schwannian stroma-poor tumors having a higher mean PI and ganglioneuroblastomas (lower mean PI).

Comparison of the PI between untreated and pretreated patients, did not show any significant differences (median PI=37.8 vs 32.0; p=0.43). The highest median PI, found in the group of untreated tumors, was correlated with advanced stages and tumor size >5 cm. Borderline correlation was observed between proliferation activity and the increase in serum LDH. Higher PI values in the group of pretreated were associated with improved response (Tab. 2).

Tumor microvascular density (MVD). The distribution of blood vessels in neoplastic tissue was heterogeneous, with vascular-rich and almost avascular fields in the same tumor. MVD was counted in "hot spots". The number of vessels ranged from 41/mm^2 to 385/mm^2 (median 164/mm^2). Analysis of the MVD in specimens from pretreated and untreated tumors indicated no significant differences (median 172/mm^2 vs 157/mm^2). Stroma-rich and stroma-poor tumors did not differ significantly with respect to vascularity (p=0.52).

In the untreated group significantly (p=0.0002) higher median MVD was found in infant tumors than in tumors from older patients (223/mm^2 vs 120/mm^2) and correlated with high serum LDH levels. No relationship was found between the microvascular density and such clinical features as the tumor stage or size in the whole group of patients, but the association between median MVD and these two clinical parameters was noticed in the pretreated group. No significant differences was seen in median MVD assessed in tumors from patients with poor outcome in comparison to the tumors from patients with an outcome in complete remission (Tab. 3).

Correlation of PI and MVD. Inverse correlation was found between PI and MVD in tumor specimens, however without statistical significance (p=0.24**)** (Fig. 2). In spite of this fact 18 tumors exhibited a high activity of both MVD and PI, while in 6 patients the activity of those two factors was very low. We calculated the value of PI x MVD, which we identified as the proliferative-vascular index (PVI). The values of PVI varied from 213 to 18333 (mean 4792, median 4179).

Table 1. Clinical data

Variable		Number of patients		
		Untreated	Pretreated	Total
Patients		37	37	74
Gender	Male	23 (60%)	26 (75%)	49 (65%)
	Female	14 (40%)	11 (25%)	25 (35%)
Age	<1 Yr	19 (50%)	10 (27%)	29 (39%)
	>1 Yr	18 (50%)	27 (73%)	45 (61%)
Stage	I	4 (11%)	0 (0%)	4 (5%)
	II	5 (16%)	0 (0%)	5 (21%)
	III	9 (24%)	11 (30%)	20 (27%)
	IV	14 (37%)	23 (62%)	37 (49%)
	Ivs	5 (13%)	3 (8%)	8 (11%)
Bone metastases In stage IV	Present	11 (79%)	15 (65%)	26 (35%)
	Absent	2 (21%)	8 (35%)	10 (15%)
Primary tumor localization	Adrenal	20 (53%)	28 (76%)	48 (64%)
	Retroperitoneal	12 (32%)	7 (19%)	19 (25%)
	Mediastinal	4 (13%)	1 (3%)	5 (8%)
	Unknown	1 (2%)	1 (3%)	2 (3%)
Largest diameter of primary tumor	<5 cm	14 (37%)	12 (32%)	26 (35%)
	>5 cm	22 (61%)	24 (65%)	46 (63%)
	Unknown	1 (5%)	1 (2%)	2 (3%)
Plasma LDH	N	11 (32%)	7 (19%)	18 (25%)
	>1x N	14 (38%)	12 (32%)	26 (35%)
	>2x N	11 (30%)	18 (49%)	29 (39%)
	Unknown	0 (0%)	1 (3%)	1 (1%)
Plasma ferritin	N	10 (26%)	11 (30%)	21 (28%)
	>N	14 (37%)	17 (46%)	31 (41%)
	Unknown	13 (37%)	9 (24%)	22 (31%)
Treatment response	CR	32 (86%)	18 (49%)	50 (68%)
	PR	0 (0%)	1 (2%)	1 (1%)
	DOD	5 (14%)	18 (49%)	23 (31%)

N – normal level, CR – complete remission, PR – partial remission, DOD – death of disease

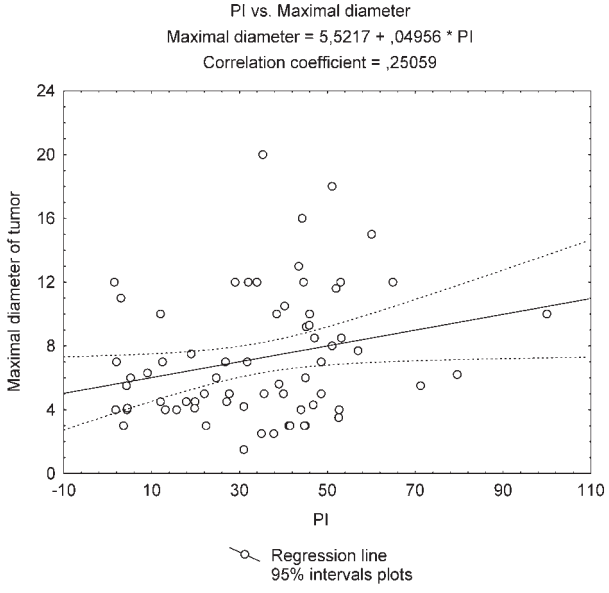

Figure 1. Correlation between proliferative activity and tumor diameter.

Table 2. Relationship between clinical factors and proliferative activity (PI) of neuroblastoma tissues

Clinical features		Untreated samples (37)		Pretreated samples (37)		Total samples (74)	
		Median PI (%)	p	Median PI (%)	p	Median PI (%)	p
Age	>1 YR	37.12	0.47	32.00	0.47	39.25	0.66
	<1 YR	33.55		38.10		35.15	
Stage	I	38.40	0.13	–	0.97	38.40	0.97
	II	22.40		–		22.40	
	III	42.30		32.00		40.30	
	IV	43.75		34.00		38.50	
	Ivs	24.85		39.66		31.00	
Largest tumor Diameter	<5 cm	27.10	0.06	24.45	0.22	27.10	0.03
	5–10 cm	40.3		41.85		40.30	
Serum LDH	N	34.40	0.07	18.95	0.06	29.35	0.03
	>1x N	33.55		4.50		29.00	
	>2x N	43.90		43.00		43.90	
Serum Ferritin	N	37.80	0.24	41.20	0.15	39.50	0.16
	>N	26.95		30.50		28.05	
Treatment response	CR/PR	35.60	0.36	46.80	0.06	39.50	0.12
	DOD	44.60		24.50		32.00	

N – normal level, CR – complete remission, PR – partial remission, DOD – death of disease

Table 3. Relationship between clinical factors and microvascular density (MVD) of neuroblastoma tissues

Clinical features		Untreated samples		Pretreated samples		Total	
		Median MVD (/mm^2)	p	Median MVD (/mm^2)	p	Median MVD (/mm^2)	p
Age	>1 YR	120	0.0007	148	0.37	121	0.0002
	<1 YR	223		129		194	
Stage	I	219	0.77	–	0.82	219	0.35
	II	173		–		173	
	III	132		208		202	
	IV	133		105		132	
	Ivs	170		106		166	
Largest tumor Diameter	<5 cm	173	0.22	179	0.17	173	0.66
	>5 cm	136		117		132	
Serum LDH	N	252	0.01	150	0.84	185	0.1
	>1x N	148		167		152	
	>2x N	132		117		121	
Serum Ferritin	N	156	0.63	151	0.41	154	0.41
	>N	139		105		133	
Treatment response	CR/PR	143	0.32	117	0.64	141	0.47
	DOD	212		168		185	

N – normal level, CR – complete remission, PR – partial remission, DOD – death of disease

PVIs greater than 7000 were found in 18 tumors (24%). Seven of these 18 tumor specimens were from infants with mostly good outcome. Among 11 patients above 1 year of age with a PVI >7000, seven (64%) died within 3–49 months (mean 22 months) (Tab. 4).

Disscusion

The prognosis of NB patients relies on well-established clinical and molecular patterns, but there are still recognized NB tumors, which have an unpredictable course. The proliferative activity as well as the angiogenic patterns plays an important role in tumor progression. The proliferative index has been documented as one of important prognostic indicators in NB as well as in many other human malignancies [4, 14, 25, 26]. Proliferative activity of neuroblastomas is heterogeneous within different tumors as well as within the same tumor [27]. Due to this heterogeneity, determining the PI from "hot spots" has been accepted. Our findings confirmed results of previous studies showing the diversity in proliferative activity of neuroblastoma tumors with the median PI=33.5. KRAMS et al documented the influence of higher proliferative activity on a shortened disease free interval and death in the patients above 1 year of age, but not in the infants [28]. Interestingly, in our study the high values of PI, were found in tumor samples from patients who had long lasting complete remission after chemotherapy. This finding can be explained by number of tumors from infants (29/74) in our study, most of which had higher PI and good prognosis. High proliferative activity proved in infant tumors had no influence on their outcome. It suggests that the remission in infants depends on the other mechanisms than the proliferative activity of tumor. It was documented earlier, that processes inducing apoptosis plays the main role in favorable outcome of infants with NB [29].

The influence of antecedent chemotherapy on tumor PI is not established definitely. KRAMS et al did not find such influence and it is in concordance with our observations. In the analysis performed by MEYA et al however, the evidence of lower PI after chemotherapy was documented [11]. In comparison in our analysis of proliferative activity as measured with PI in 59 Schwannian stroma-poor tumors was not significantly lower in specimens from pretreated patients in comparison to untreated ones. This observation is in agreement with our previous studies in a smaller group of NB patients [9]. It ap-

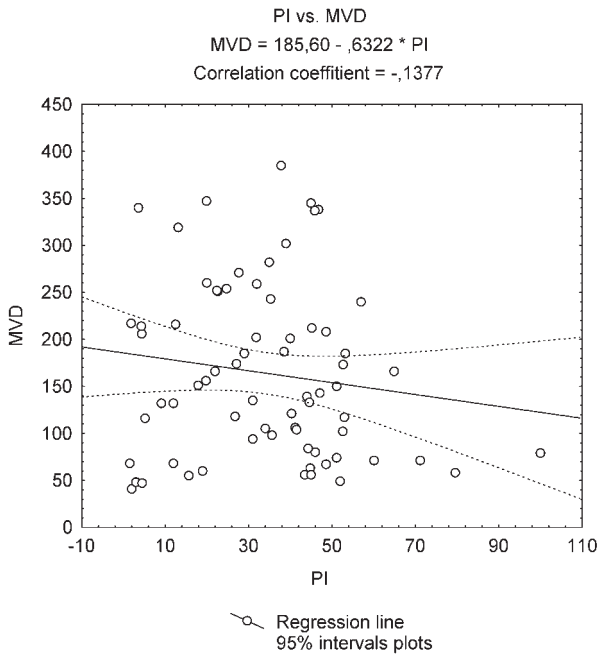

Figure 2. Correlation between proliferative activity and vascular density in NB.

pears that different results could depend on the different characteristics of the patient groups studied. In studies performed by KRAMS the highest PI was noticed in disseminated tumors whereas our analysis does not confirm that observation [28]. The present study showed significant associations of larger tumor size with higher PI. We interpreted this correlation as the obvious consequence of high proliferative activity resulting in increased tumor volume. However no relations between PI and the stage of disease were found.

The relationship between morphologic types and proliferative activity, with lower PI in ganglioneuroblastomas than in stroma-poor NB was observed also by other authors [6, 11].

The research on angiogenesis in NB gives the insight into biology of this tumor [20]. The prognostic signicance of this parameter was demonstrated in NB by MEITAR et al, who reported correlation of MVD with metastatic disease and poor clinical outcome [21]**.** Some authors showed the linkage of higher MVD with clinical stage [10, 22]. In our investigation we analyzed the relationship between the proliferation index and vasculature of NB tumors with some clinical features. Studies performed by CANETE did not confirm the association of vascular density with clinical prognostic parameters and survival [30]. The comparison of our results with the other studies is difficult due to different methodology and the way of presentation of the results. However our methodology was the closest to the study by CANETE et al [30]. In our investigation we analyzed the relationship between the proliferation index and vasculature of NB tumors with some clinical features. We did not find any correlation between MVD and

Table 4. The highest values (>7000) of proliferative-vascular index (PVI) in relation to the course of disease

No of patients	Age	PVI	Stage			Outcome	
			I, II, IVs	III	IV	CR	DOD
7	<1YR	7322 – 18333	5	1	1	6	1
11	>1 YR	7200 –15468	0	3	8	8	3

N – normal level, CR – complete remission, PR – partial remission, DOD – death of disease

clinical factors, however tumors from patients with poor outcome exhibited higher MVD. Such divergences could arise from the heterogenous characteristics of NB tumors.

Relationship between proliferation and vascularity suggests that in some cases the angiogenesis couldn't follow the proliferation of tumor. The observation that some tumors presented having high PI in association with high MVD stimulated us to propose the new parameter, PVI. In 4/11 infant tumors, high values of PVI had no influence on the patient's outcome. However we observed quick progression and poor outcome in 7/11 (64%) patients above 1 year-old with a high PVI in tumors. This observation needs to be confirmed in further, larger studies.

Our study revealed the statistically significant correlation between the PI and the patients' age, tumor diameter and tumor morphology. Moreover NB tumors demonstrated a tendency to have a inverse relationship between proliferative index and angiogenesis intensity measured with MVD. We observed however the coexistence of high proliferative activity with high microvascular density in some patients above 1 year of age. Thus high PVI is connected with more aggressive course of disease. The prognostic value of simultaneous estimation of MVD and PI as PVI requires confirmation in larger studies.

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