ОРИГИНАЛЬНЫЕ ИССЛЕДОВАНИЯ

DIAGNOSTIC VALUE OF MICROVESSEL STRUCTURE IN BRAIN GLIAL TUMORS

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Diffuse gliomas are the most common primary brain tumors with a disproportionately high mortality rate. Characteristics of microvessels are of high diagnostic and prognostic significance, however, the results of previous studies are controversial. The aim of the work is to evaluate the features of angiogenesis in diffuse gliomas on the basis of determining the qualitative and quantitative microvascular characteristics. Also important is their relationship with the histological type of tumor. Microvascular density (μm^{-1}), total vascular area (%), total lumen area (%) and the mean diameter of microvessels (μm) were measured and calculated in diffuse brain gliomas (n=76) using GFAP-negative status of endothelium in the presence of exclusively GFAP-positive tumor cells. Proliferation of microvessels was evaluated using proliferation index of vascular epithelium (Ki-67). The possibility of routine evaluation of the angiogenesis in diffuse gliomas using GFAP and Ki-67 markers was defined. We revealed significant correlation between features of the neoplastic microvasculature and WHO Grade.

Keywords: *brain tumor, immunohistochemistry, diagnostics, proliferation of vessels, density of vessels.*

Diffuse gliomas are the most common primary brain tumors with extremely high mortality rate and tumor cells, that display signs of astrocytic and/or oligodendrocytic differentiation. WHO classification of CNS tumors (4th revised edition, 2016) introduced the "integrated" diagnosis that based on histological and molecular peculiarities of a neoplasm [1]. First of all, the histological variant of glioma is determined on the basis of phenotypic characteristic of distinct diagnostic categories using both routine method of staining and immunohistochemical (IHC) technique [2]. (Secondly, molecular features are estimated and added). Traditionally, proliferative activity of neoplastic cells, cellular and nuclear pleomorphism, evidence and spread of secondary tumor alterations are the most important criteria for evaluation of tumor type and Grade [3].

In diffuse gliomas, the four basic morphologic types of microvessels are distinguished depending on their structure: 1) glomeruloid type – a group of vessels surrounded with connective-tissue stroma; 2) vascular "garlands" – vessels with or without connective-tissue stroma arranged into garland-like structures mostly localized around tissue with necrotic alterations; 3) vessel clusters – separate regions of microvessels (≥3) of bizarre shape without connective-tissue stroma; 4) capillary-like vessels – uniformly distributed thin microvessels resembling normal brain capillaries [4, 5].

There are several references to diagnostic significance and independent prognostic significance of such angiogenesis parameters as microvascular density [6-8], total microvascular area (%) [9], morphological type of vessels [4, 5]. Moreover, there is certain relation between microvascular features structure and other morphological and clinical signs of tumors [10].

IHC methods simplify and objectify microvasculature examination of vessels. CD34, CD31, von Willebrand factor are the most commonly used markers for the microvessel labeling. Therefore, the aim of this study was to evaluate the microvasculature features in diffuse gliomas on the basis of determining the qualitative and quantitative

microvascular characteristics imunohistochemically and to establish their relationships with the histological type of tumor.

Materials and Methods

Patients. 76 patients (tab. 1) with glial tumors were included in investigation. Samples of gliomas were obtained with surgery in the second neurosurgery department of Dnipropetrovsk Regional Clinical Hospital in 2006-2016. Histological diagnosis was made on the basis of modern histological and immunohistochemical criteria [3].

Table 1

Patients Characteristics (n, %)

	Number of cases (n)	%
Gender		
male	36	47,4
female	40	52,6
Age		
≤50	48	63,2
>50	28	36,8
Histological diagnosis		
diffuse astrocytoma (DA) – Grade II	10	13,2
anaplastic astrocytoma (AA) – Grade III	14	18,4
glioblastoma (G) – Grade IV	36	47,4
oligodendroglioma (O) – Grade II	8	10,5
anaplastic oligodendroglioma (AO) – Grade III	8	10,5
Total	**76**	**100%**

IHC. Besides the routine histological examination (hematoxylin-eosin staining), the immunohistochemical analysis was performed according to TermoScientific (TS) protocols for GFAP (RTU; DakoCytomation, Denmark) and Ki-67 expression (clone sp6, 1: 400; TS, USA). Visualization system Lab Vision Quanto (TS, USA) was used with detection of the protein chain using DAB Quanto Chromogen (TS, USA) for cut-off with 4 μm thickness.

Morphometric study. Morphotype of vessels, their quantity, areas and its diameters were determined on the basis of the absence GFAP in the endothelium with strong (+++), moderate (++) or weak (+) GFAP-immunoreactivity of the surrounding neoplastic cells. Digital photos were obtained from the regions of studied tumors using ZEISS Axiocam 105 color camera under Axio Scope.A1 microscope (magnification x400). Each sample was illustrated with 3 photos from the regions with the highest microvessel density. The area and linear dimensions were measured using instruments of ImageJ 1.49v package [11].

The measured parameters were used for calculation of microvascular density (per 1 mm^2 of tumor area), total vascular area (%), total lumen area (%), mean diameter of microvessels (in μm) [12, 13]. Proliferative activity of microvessels was evaluated with endothelial proliferative index – the ratio of the quantity of Ki-67-immnoreactive endothelial nuclei to their total quantity expressed in percent [12].

Statistical analysis was conducted using STATISTICA software (version 6.1; serial number AGAR 909 E415822FA). Shapiro-Wilk test was used for checking of normal distribution of the values. Statistical significance was determined by Kruskal-Wallis test, that allow to compare characteristics of the studied groups (n=5) with subsequent determination of Mann-Whitney criteria. Correlative analysis was performed with Spearman's rank. The value $p<0,05$ was assumed to be statistically significant [14].

Results and Discussion

To conduct more accurate morphometric examinations, hidden capillaries were detected by the absence of GFAP vascular accumulation and exclusively GFAP-positive cytoplasm of surrounding neoplastic cells (fig. 1A-B). It should be noted that the examined parameters was similar ($p>0,05$) in normal brain tissue and infiltration zone.

In Grade II tumors (DA and O), the capillaries were mostly detected that phenotypically did not differ from normal ones (94%). In AA and AO (Grade III), more frequent budding was noted that influenced the quantity of vessels resembling normal ones by form (78%). Glioblastomas (Grade IV) showed intense angiogenesis which lead to formation of garland-like structures in 76% of samples, with glomeruloid vessels found only in 18% of samples; vessels resembling normal capillaries of the brain were in 37% of tumors.

In figure 2 the mean values of morphometric vascular parameters in diffuse gliomas are given. In different forms of diffuse gliomas, reliability of differences was assested with Kruskal-Wallis test (the microvascular density, total vascular area, total lumen area and endothelial proliferative index). Using Mann-Whitney test it was found that values of microvascular density and total vascular area were significantly lower in DA and O (Grade II) than in Grade III-IV tumors ($p<0.01$). Also, no reliable differences were revealed between astrocytic and oligodendroglial tumors with the identical Grade and among Grade III-IV tumors ($p>0.05$). Similar dependences were found in the analysis of total lumen area, however, the lowest values of this parameter were recorded in AO ($p<0.01$). The highest total lumen area were recorded in G, in other histological forms of diffuse gliomas this parameter had intermediate and statistically similar values. Endothelial proliferative index significantly varied in tumors of different Grade. Thus, tumors referred to Grade II, demonstrated the lowest endothelial proliferative index, glioblastomas showed maximal values, and Grade III diffuse gliomas – intermediate values ($p<0.05$). The mean diameter varied in tumors with different histological structure within the range of statistical error (Kruskal-Wallis test, $p=0.069$).

The following parameters showed direct significant correlation with Grade (WHO): microvascular density ($r=0.596$), total vascular area ($r=0.275$), total lumen area ($r=0.813$) and endothelial proliferative index ($r=0.746$).

Correlative analysis showed a moderate direct strong link between the microvascular density, endothelial proliferative index and total vascular area. The mean diameter of microvessels did not correlate with any of the studied parameters (Spearman coefficient had no statistical significance).

Immunohistochemical markers of the vascular wall (CD34, CD31) were widely used in previous studies of the microvasculature features in diffuse gliomas as well as in other solid tumors [4, 5, 7-9, 12]. However, these markers are rarely used in routine pathological practice for parenchymatous brain tumors, therefore the authors used negative staining of the vascular wall for morphometric measurements. Here, GFAP approved as the best one – it's strong or moderate expression was observed by tumor cells with the absolute absence of the respective protein in the vascular walls in all diffuse gliomas [3].

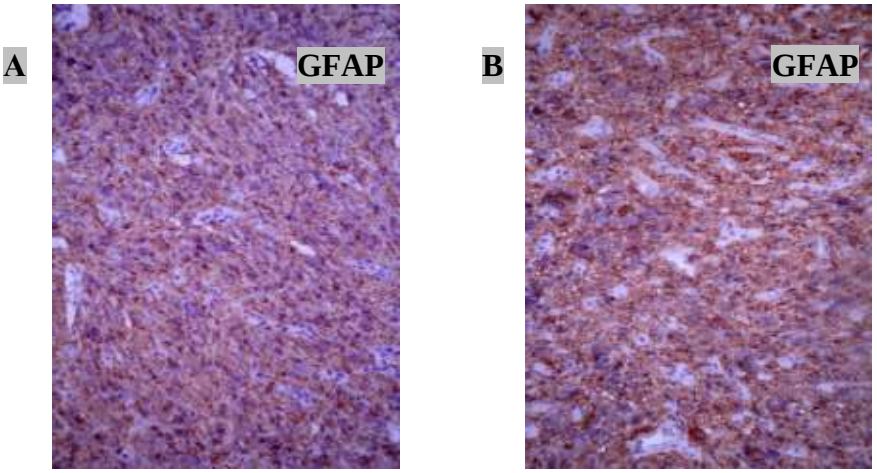

Fig. 1. Diffuse (A) and anaplastic (B) astrocytomas. GFAP does not appear within the vessels, though it is expressed in the surrounding tumor tissue. IHC, hematoxilin counterstaining, ×400

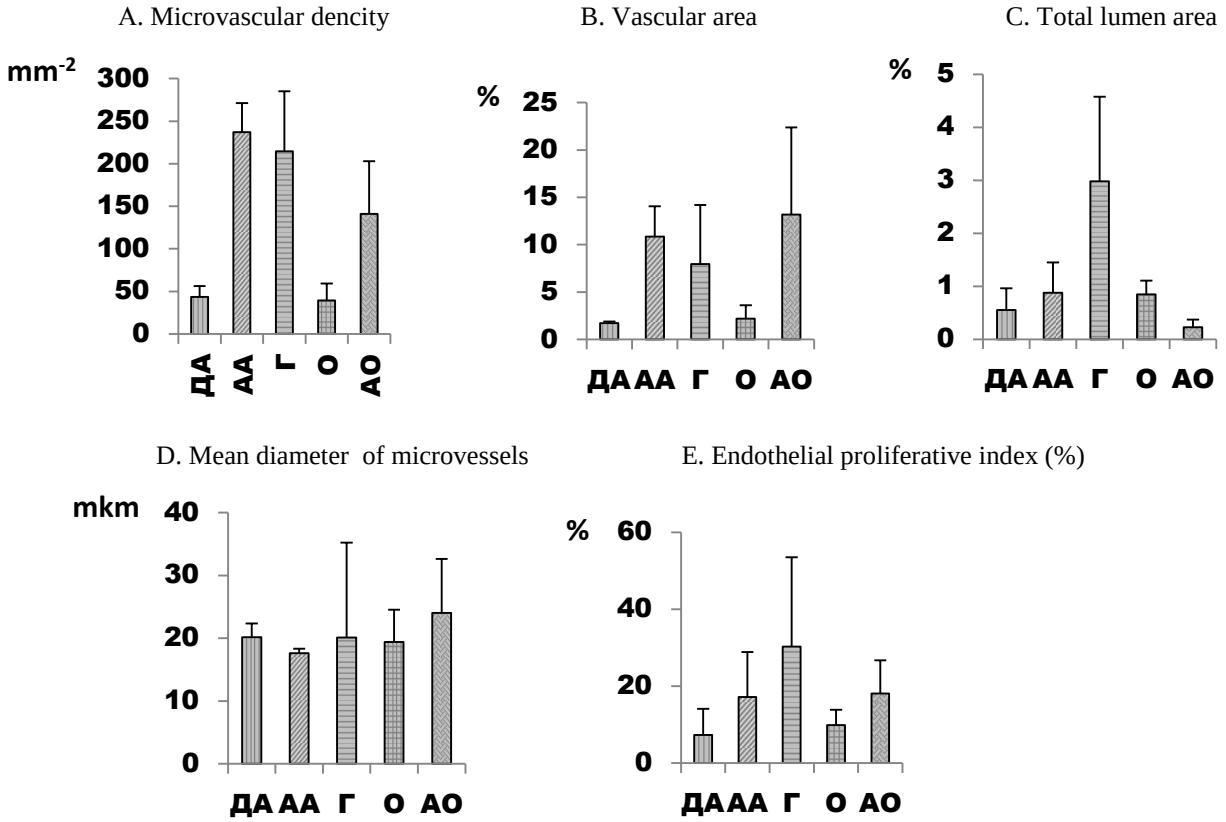

Fig. 2. Quantitative characteristics of microvasculature in diffuse brain gliomas. A. Microvascular density (mm^{-2}). B. Total vascular area (%). C. Total lumen area (%). D. Mean diameter of microvessels (μm). E. Endothelial proliferative index (%)

Morphometric results showed dependence of the angiogenesis intensity on Grade of gliomas. Here, increased quantitative parameters (microvascular density, endothelial proliferative index, etc.) induces changes in the qualitative characteristics of tumor vessels: formation of cascade of microvessels ("garlands") and glomeruloid structures in highly malignant neoplasms [5, 9].

Hypoxia and pseudohypoxia are considered significant triggers of vessel growth in diffuse gliomas. Pseudohypoxia is caused by metabolic changes associated with mutation of isocitrate dehydrogenase gene, which is a primarily characteristic for low-malignancy gliomas. It must be said that hypoxia is more evident in polymorphic neoplasias with high cell density (Grade III-IV), being the most potent stimulus.

Conclusions

1. GFAP does not appear within the walls of vessels, but strictly counterstains the surrounding tumor tissue. This allows to carry out the evaluation of microvasculature in diffuse gliomas by measurements of GFAP-negative areas.

2. The statistically significant relationship is determined between endothelial proliferative index (Ki-67) and Grade WHO ($r=0.746$, $p<0.05$).

3. Diffuse astrocytomas and oligodendro-gliomas (Grade II) are characterized by uniform capillary-like microvessels, garlands of micro-vessels that may be noted in Grade III-IV tumors, and glomeruloid vessels that may be present in glioblastomas (Grade IV).

4. The microvessel density, total vascular area and total lumen area correlate directly with the Grade WHO. Microvascular density and total vascular area are significantly higher in Grade III-IV gliomas than in diffuse astrocytomas and oligodendrogliomas (Grade II), $p<0.01$.

Authors have no conflict of interest to declare.

References

1. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK et al. The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol*. 2016; 131 (6): 803-20.

2. Shynkarenko TV, Shponka IS, Kornilov BY. Alghorytmy diaghnostyky astrocytarnykh pukhlyn gholovnogho mozku u doroslykh z urakhuvannjam pereghljadu klasyfikaciji pukhlyn centraljnoji nervovoji systemy [Diagnostic algorithms for astrocytic brain tumors in adults according to revised classification of nervous system tumors]. *Morphologia*. 2016; 10 (3): 46-52. (in Ukrainian).

3. Louis DN, Ohgaki H, Wiestle OD, Cavenee WK, eds. *WHO classification of tumours of the central nervous system*. Revised 4th ed. Lyon: IARC; 2016. 408 p.

4. Birner P, Piribauer M, Fischer I, Gatterbauer B, Marosi C, Ambros PF et al. Vascular patterns in glioblastoma influence clinical outcome and associate with variable expression of angiogenic proteins: evidence for distinct angiogenic subtypes. *Brain Pathol*. 2003; 13 (2): 133-43.

5. Chen L, Lin ZX, Lin GS, Zhou CF, Chen YP, Wang XF et al. Classification of microvascular patterns via cluster analysis reveals their prognostic significance in glioblastoma. *Hum pathol*. 2015; 46 (1): 120-8.

6. Cai H, Xue Y, Liu W, Li Z, Hu Y, Li Z et al. Overexpression of Roundabout4 predicts poor prognosis of primary glioma patients via correlating with microvessel density. *J Neurooncol*. 2015; 123 (1): 161-9.

7. Clara CA, Marie SK, Almeida JR, Wakamatsu A, Oba-Shinjo SM, Uno M al. Angiogenesis and expression of PDGF-C, VEGF, CD105 and HIF-1α in human glioblastoma. *Neuropathology*. 2014; 34 (4): 343-52.

8. Yao Y, Kubota T, Takeuchi H, Sato K. Prognostic significance of microvessel density determined by an anti-CD105/endoglin monoclonal antibody in astrocytic tumors: Comparison with an anti-CD31 monoclonal antibody. *Neuropathology*. 2005; 25 (3): 201-6.

9. Korkolopoulou P, Patsouris E, Kavantzas N, Konstantinidou AE, Christodoulou P, Thomas-Tsagli E et al. Prognostic implications of microvessel morphometry in diffuse astrocytic neoplasms. *Neuropathol appl neurobiol*. 2002; 28 (1): 57-66.

10. Shi J, Zhao Y, Yuan Y, Wang C, Xie Z, Gao X et al. The expression of IDH1 (R132H) is positively correlated with cell proliferation and angiogenesis in glioma samples. *Chinese journal of cellular and molecular immunology*. 2016; 32 (3): 360-3.

11. Poslavska OV. Metodologhija vykorystannja proghramnogho zabezpechennja dlja analizu cyfrovykh mikrofotoghrafij na bazi kursu patomorfologhiji z metoju pidvyshhennja profesijnogho rivnja studentiv i naukovciv [Methodology for the use of software for the analysis of digital micrographs on the base of pathomorphology course in order to increase the professional level of students and scientists]. *Morphologia*. 2015; 9 (3): 122-6. (in Ukrainian).

12. Mittelbronn M, Baumgarten P, Harter PN, Plate KH. *Analysis of cerebral angiogenesis in human glioblastomas*. In Cerebral Angiogenesis: Methods and Protocols. New York. 2014; 187-203.

13. Potts SJ, Eberhard DA, Salama ME. *Practical approaches to microvessel analysis: hotspots, microvessel density, and vessel proximity*. In Molecular Histopathology and Tissue Biomarkers in Drug and Diagnostic Development. New York. 2015; 87-100.

14. Antomonov M. *Matematicheskaja obrabotka i analiz mediko-biologicheskih dannyh* [*Mathematical processing and analysis of biomedical data*]. K.: Firma Maliy Druk; 2006: 381-91. (in Russian)

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