SPECTRUM OF CONCOMITANT BRAIN VASCULAR LESIONS IN PEDIATRIC DIFFUSE GLIOMAS

Abstract. Cerebrovascular disease represents a threatening factor for brain cancer survivors. However, a comprehensive evaluation of small vessel disease related to gliomas has not yet been performed.

This study aims to characterize concomitant vascular lesions in pediatric diffuse gliomas and identify their association with the molecular subgroup of tumors.

We performed a retrospective pathological study of biopsy samples of 77 pediatric patients with diffuse gliomas, treated in Belarusian Research Center for Pediatric Oncology, Hematology and Immunology. Eight molecular subgroups were identified by immunohistochemical and cytogenetic studies (H3K27mut, ALT, IDH1mut, BRAFmut-PXA, FGFR1, BRAFmut/FGFR2, RTK, MYB). In each group microvessel density/area (MVD/MVA), tumor vessels co-option and signs of small vessels disease (SVD) were determined.

The levels of microvascularization significantly differed between the molecular subgroups of diffuse gliomas, indicating the presence of intrinsic pro-angiogenic activity there. The highest values of MVD/MVA, as well as rate of hemorrhagic necrosis, were found in the BRAFmut/FGFR2, RTK groups. SVD was common in the adjacent tissues of gliomas and occurred in 32.5 % of cases. High grade SVD was associated with the BRAFmut/FGFR2 and IDH1mut subgroups. BRAFmut/FGFR2 tumors were more aggressive and caused cortical microinfarctions in 84.6 % and leukoaraiosis in 87.5 % of cases. IDH1mut tumors were mainly linked with cortical microinfarctions (60 % of cases).

The results of the study suggest that concomitant small vascular lesions are common in adjacent tumor tissue and can significantly influence the overall rate of cerebrovascular disease in convalescents with diffuse gliomas.

Keywords: pediatric diffuse gliomas, angiogenesis, small vessels disease, BRAF mutation, IDH1 mutation


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и вызывают микроинфаркты в 84,6 % случаях и лейкоареоз в 87,5 % случаях. Для опухолей IDH1mut подгруппы более характерно развитие микроинфарктов в коре (60 % случаев).

Результаты исследования показывают, что сопутствующие сосудистые поражения часто встречаются в перифокальной опухолевой ткани и могут значительно влиять на риск возникновения цереброваскулярных заболеваний у реконвалесцентов с диффузными глиальными опухолями.

**Ключевые слова:** диффузные глиомы у детей, ангиогenez, болезнь мелких сосудов, мутация BRAF, мутация IDH1


**Introduction.** The incidence of cerebrovascular diseases is higher in brain cancer patients than in the general population, significantly aggravating their condition and prognosis [1, 2]. As a result of radiation therapy or chemotherapy or due to cancer itself, brain cancer patients have concomitant cerebrovascular diseases [3], which subsequently cause more frequent cognitive disorders, strokes and vascular dementia. Patients with cancer have been shown to have higher in-hospital post-stroke mortality rate [1, 4, 5] and patients with ischemic stroke with an active cancer have also been found to be of younger age, with more severe and more frequent cryptogenic strokes [1].

Small vessel disease (SVD) is the functional basis of cerebrovascular diseases. SVD is a complex pathology of vessel’s wall caused by both environmental and genetic factors. Undoubtedly, the tumor occurrence could be considered as an imbalance of genetic homeostasis. And the role of oncogenes in promoting neovascularization has recently gained considerable attention. Oncogenic mutations are implicated in the activation of the ‘angiogenic switch’ in tumors. Some of the oncogenes can promote tumor angiogenesis. In addition, the tumor indirectly causes oxidative stress and inflammation that are suspected to be crucial for vascular damage.

Direct tumor effects on angiogenesis vary considerably, and include hyperplasia of tumor vessels due to angiogenesis, vascular cooption, vascular mimicry, arterial and venous sinus invasion by tumor mass or leptomeningeal infiltrates, blood vessel compression by tumor growth or tumor bed edema, disruption of blood-brain permeability and rarely the development of vascular malformations. SVD can be viewed as a special case of disruption of blood-brain barrier [6].

The present study focuses on the possible molecular mechanisms and causes of small vessel disease in brain cancer patients, and aims to identify the most common molecular type of pediatric glial tumor causing small vessel disease.

**Objects and research methods.** We enrolled 77 consecutive patients with diffuse gliomas treated in Belarusian research center for pediatric oncology, hematology and immunology from January 2015 till June 2021. All gliomas were diagnosed on the basis of pathological criteria, and all slides from the resected specimens, including those used for immunohistochemistry, were reassessed independently by two neuropathologists. In order to exclude the effect of radiation therapy, we studied the vascularization of tumor and peritumoral tissue only on biopsy material before any treatment.

To gain insights into molecular groups of diffuse pediatric gliomas, we performed an integrated analysis of expression of immunohistochemical markers (H3K27me3, IDH1-R132H, ATRX, p53, BRAF-V600E, Olig2, GFAP, EMA, pan-TRK), and fusions affecting genes that encode receptor tyrosine kinases (FGFR2, ALK, ROS, NTRK1), focal copy-number alterations (CDKN2A/B and 1p/19q) and identified several molecular subgroups of pediatric glial tumors – H3K27mut, ALT, IDH1mut, FGFR1, BRAFmut-PXA, BRAFmut/FGFR2, RTK, MYB. In these groups, we studied tumor angiogenesis by assessing the level of microvessel density and microvessel area, the presence of tumor vessels co-option and the pathological signs of SVD.

**Immunohistochemistry.** Tumor tissue obtained during surgery was fixed in 10 % buffered-formalin, routinely processed, paraffin-embedded, sectioned at 5mm, and stained with hematoxylin and eosin. Representative paraffin blocks were selected for immunohistochemistry (IHC) studies. Immunohistochemistry was performed on 2.5-lm-thick paraffin sections following heat-induced epitope retrieval using CC1 (Ventana), then staining with GFAP (Leica Biosystems), Olig2 (Elabioscience), ATRX (Elabioscience), p53 (Ventana), synaptophysin (Leica Biosystems), VE1 (Ventana), neurofilament (Cell Marque), epithelial membrane antigen (Cell Marque), CD34(Ventana), and Ki-67 (Cell Marque) on a Ventana Benchmark XT automated stainer, using Ventana UltraView Universal DAB Detection kits and OptiView DAB Detection kits.
**Fluorescence in situ hybridization.** On touch preparations, cells were fixed directly with a 9:1 mixture of methanol and acetic acid for 15 min and air-dried overnight. After enzymatic pepsin treatment, nuclear DNA was denatured in 70% formamide/2×SSC and dehydrated. Hybridization of the probe and post-hybridization washing were carried out in accordance with the manufacturer’s recommendations. CDKN2A homozygous deletion was analyzed using a commercially available locus-specific probe (9p21, Abbott Molecular/Vysis) and centromere 9 (CEP9) control probe. The status of the FGFR2 and RTK genes was assessed using fluorescent in situ hybridization (FISH) using two-color ZytoLight SPEC FGFR2 Dual Color Break Apart Probe, Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular, Abbot Park, IL, USA), ZytoLight® SPEC ROS1 Break Apart Probe (ZytoVision, GmbH).

**Statistical analysis** was performed using R software (version 3.4.4). All data were presented as median for continuous variables and number (percentage) for discrete variables. Group comparisons were made using the Mann–Whitney U nonparametric single factor test. Fisher’s exact test was performed for categorical variables.

**Neuropathological assessment of SVD** in the brain tissue included evaluation of progressive vasculopathy and parenchymal lesions in the cortex and white matter. There are definitions of neuropathological descriptive terms as follows. By definition, small vessel changes involve hyalinization of vessels, expansion of the perivascular space, tortuosity, and pallor of adjacent perivascular myelin, with associated astrocytic gliosis. Lacunar infarct means complete or cavitation lesion in both subcortical gray and white matter. White matter changes focus on venous collagenosis and leukoaraiosis. Venous collagenosis – the thickening of the walls of periventricular veins and venules by collagen. Leukoaraiosis comprises several patterns including myelin pallor or swelling, tissue rarefaction associated with loss of oligodendrocytes, diffuse axonal injury with thinning and varicosities, loosening of axon–oligodendrocyte adhesion and gliosis. Cerebral microinfarction is the accumulation of small, even miniscule, ischemic lesions with or without a small vessel at its center but with pallor, neuronal loss, axonal damage and gliosis.

**Microvessel density and microvessel area assessment.** To quantify the vascular density three tumor areas with the highest vascular density were selected in each tumor independently from the tumor cell density, using a microscope (Nikon Eclipse E550i) equipped with a digital camera and Nis-Elements BR 2.30 software. Microphotography of CD34 immunolabeling was acquired at high power fields (0.125 mm², ×200) for the three areas. The total number of vascular sections in the three pictures was obtained by visual count. Every immunopositive structure (endothelial cell or cell cluster) clearly separated from neighboring microvessels, neoplastic cells, or other connective tissue elements, was treated as a microvessel. Vessels with visible muscle layers were excluded from analysis as these are not classified as microvessels. The MVD was defined as the mean number of microvessels in the three most vascularized fields of view per 1 mm². The microvessel’s area was defined as the mean area of cross-sectional areas of microvessel lumen in the three most vascularized fields of view, calculated in pixels.

**Research results.** In all studied cases of tumors, quantitative parameters of microvascularisation were increased comparing to normal samples of brain (MVD – 48 microvessels/mm², MVA – 2810 pixels/mm²). In addition to high rate of the vascular branches, some tumors were characterized by calcification of the vessel wall and the precipitation of calcium crystals into the perivascular space, they were found in 12 cases (Tab. 1). Evidence of tumor co-option of vessels were found in 60 cases, the presence of tumor satelliosis was nonspecific and was noted in all groups of gliomas. Specifically the presence of growth in the perivascular spaces was significantly more frequent in the BRAFmut-PXA and MYB subgroups. The presence of these changes may indicate a possible venous outflow disturbance in the peritumoral zone.

In 6 cases, calcification was pronounced, calcifications merged with each other, hemorrhages and hemorrhagic necrosis were detected around. Hemorrhagic necrosis and hemorrhages were more common in gliomas of BRAFmut/FGFR2 and RTK subgroups and occurred in 30.8 and 50% cases respectively. The formation of hemorrhagic necrosis around these vessels indicates their immaturity and functional failure, leading to permeability of the brain-blood barrier. It should be noted that intratumoral hemorrhages were also more often observed in this group, which has important clinical significance, since, on the one hand, they can cause disability and even be life-threatening, on the other hand, they can be leading symptoms masquerading the presence of a tumor.
Table 1. Frequency distribution of characteristics of angiogenesis in different molecular subgroups of diffuse gliomas

<table>
<thead>
<tr>
<th>Characteristics of angiogenesis</th>
<th>Molecular subgroups of diffuse pediatric gliomas</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FGFR1</td>
<td>BRA Fmut-PXA</td>
</tr>
<tr>
<td>Vessels cooption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perivascular satellitosis, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>14 (100)</td>
<td>7 (63.6)</td>
</tr>
<tr>
<td>p = 0.0155 p = 0.4623 p = 0.7213 p = 0.4210 p = 0.5636 p = 1.0000 p = 1.0000 p = 0.5676 p = 0.0008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth in perivascular spaces, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0 (0)</td>
<td>9 (81.8)</td>
</tr>
<tr>
<td>p = 0.1096 p &lt; 0.0001 p = 1.0000 p = 0.3383 p = 0.0722 p = 0.3430 p = 0.1970 p = 1.0000 p = 0.5819</td>
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</tr>
</tbody>
</table>

Table 2. Frequency distribution of SVD and parenchymal lesions in different molecular subgroups of diffuse gliomas

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>Total</th>
<th>Molecular subgroups of diffuse pediatric gliomas</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FGFR1</td>
<td>BRA Fmut-PXA</td>
<td>BRA Fmut/FGFR2</td>
</tr>
<tr>
<td>SVD, Eziri grade 2–3, n (%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>25</td>
<td>2 (14.3)</td>
<td>0 (0)</td>
<td>11 (84.6)</td>
</tr>
<tr>
<td>p = 0.1284 p = 0.0130 p &lt; 0.0001 p = 0.0197 p = 0.0003 p = 0.6790</td>
<td></td>
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<tr>
<td>Venous collagenosis, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>0 (0)</td>
<td>1 (9.1)</td>
<td>10 (76.9)</td>
</tr>
<tr>
<td>p = 0.4920 p = 0.1546 p &lt; 0.0001 p = 0.8079 p = 0.6100 p = 1.0000</td>
<td></td>
<td></td>
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<tr>
<td>Leukoaraiosis, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2 (16.7)</td>
<td>0 (0)</td>
<td>7 (87.5)</td>
</tr>
<tr>
<td>p = 0.1696 p = 0.5455 p = 0.0009 p = 1.0000 p = 1.0000 p = 1.0000</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cortical microinfarction, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0 (0)</td>
<td>1 (9.1)</td>
<td>11 (84.6)</td>
</tr>
<tr>
<td>p = 0.0155 p = 0.2705 p &lt; 0.0001 p = 0.1032 p = 0.5336 p = 1.0000 p = 1.0000 p = 1.0000 p = 0.3188</td>
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Note. Fisher’s exact 2-tailed test was applied, p-value 0.05 considered statistically significant.

Note. * – Fisher’s exact 2-tailed test was applied, p-value 0.05 considered statistically significant, ** – Mann–Whitney U test was applied, p-value 0.05 considered statistically significant.
The level of vascular density and the area of microvessels were significantly increased in the groups of tumors with the BRAFmut/FGFR2, RTK phenotype. The distinction was confirmed by the Mann–Whitney test. Possibly, the regulation of angiogenesis in BRAFmut/FGFR2, RTK subgroups is carried out not only through the secretion of hypoxia-inducible factor-1α (HIF-1α), but also due to the co-stimulation of the VEGFR and TGFR-beta signaling pathways components by constantly activated RTK tumor signaling.

In our study, the disruption of blood-brain barrier in the peritumoral zone was widespread although severe parenchymal changes associated with SVD such as lacunae, lacunar infarctions were absent. Expansion of perivascular spaces was detected in different groups of tumors, totally in 35 cases, peri-
vascular myelin pallor – in 25 cases, microbleeds – in 36 cases. Cases were classified as small vessels disease when widening of perivascular spaces, thickening of vascular walls, accumulation of perivascular macrophages, and perivascular myelin pallor or attenuation of nerve fibres were present. For this study, case classification for SVD was based on the presence of SVD using the Eziri grading scheme. The most significant changes including pallor of myelin fibers to necrosis and cavitation were detected in 17 and in 19 cases respectively. The non-functional tortuous vessels were observed in 3 cases. The periventricular parts of the white matter are more prone to the development of venous collagenosis, however, this area was not always included in the preparation of the removed tumor and it was possible to assess this change in 45 cases. In 17 cases of them venous collagenosis was detected, in 15 cases – signs of leukoaraiosis.

Exemplificative histological features of small vessel disease are shown in the Figure.

There were differences in the severity and prevalence of SVD in different molecular groups of gliomas. Most of the severe cases of small vessel lesions (Eziri grade 2–3) occurred in the IDH1mut and BRAFmut/FGFR2 subgroups of diffuse gliomas. The association between SVD and these subgroups was statistically significant. Indeed the frequency of SVD grade 2–3 in the BRAFmut/FGFR2 subgroup was 84.6 % \( p < 0.0001 \) and in the IDH1mut subgroup – 70 % \( p = 0.0111 \). As a consequence, cortical microinfarctions most often occurred in the perifocal zone of these tumors (85 % of all microinfarctions). The lesions of the white matter of the brain – leukoaraiosis and venous collagenosis were more typical for the BRAFmut/FGFR2 subgroup of diffuse gliomas and was found in 87.5 % \( p < 0.0009 \) and in 100 % cases respectively \( p < 0.0001 \).

**Discussion.** Research in the field of molecular basis of the vascular pathology of the tumor and adjacent brain tissue is limited. Most of the articles devoted to the study of tumor vasculopathies concentrate on the development of SVD after the use of radiation therapy \[7, 8\]. The majority of authors come to an agreement that the use of radiation therapy increases the risk of stroke, dementia, and other vasculopathy in brain cancer survivors. However, none of these studies take into account the influence of the molecular alterations on the development of parenchymal lesions of SVD.

A few reports have reported the occurrence of SVD on the background of a genetic predisposition. D. Unruh et al. reported the risk of venous thromboembolism to be extremely low in patients with IDH1 mutated gliomas linked to low podoplanin and tissue factor expression \[9\].

There are also few case reports mentioning a high risk of small vessels disease in patients with neurofibromatosis 1. Apart from moya-moya phenomena they can include occlusion, aneurysm, pseudoaneurysm, ectasia, stenosis, fistula, rupture and lacunar infarcts \[10, 11\].

According to the results of our study, small vessels disease correlates with BRAFmut/FGFR2 and IDH1mut subgroups. On the one hand, the BRAFmut/FGFR2 and IDH1mut subgroups, unlike all others, are slowly growing neoplasms, and the presence of SVD there is most likely a consequence of circulatory disturbance in the brain due to mass effect, vessel infiltration by cancer cells and obstruction of the outflow of cerebrospinal fluid because of leptomeningeal growth. On the other hand, the BRAFmut/FGFR2 subgroup is characterized by a very high vascularization and a large number of fragile immature vessels, which are less common in other groups. Such vessels are more likely to undergo calcification and, accordingly, function poorly, increasing the load on the preexisting cerebral vessels, causing SVD. In IDH1mut, the mechanism of SVD formation is similar due to the presence of tortuous and non-functioning vessels \[12, 13\].

The risk group of SVD is formed by two categories of gliomas – BRAFmut/FGFR2 and IDH1mut gliomas, whose management involves surgical treatment, radiation therapy, or watch-and-wait strategy, or their combinations \[14, 15\]. Overall, our research indicates that these groups of patients may benefit from early maximum safe resection rather than watchful waiting. It can also be assumed, that the excessive use of radiation therapy for the residual tumor in these patients may lead to neurological deterioration with seizures, intellectual disability and ischemic stroke.

**Conclusion.** Vigilance for SVD is always required in patients with glial tumors. A precise diagnosis is necessary in order to avoid mistreating patients and help improve their outcome and quality of life.
Genetic studies are also important for elucidation of the risk of SVD. The results of our research allow us to conclude that BRAFmut/FGFR2 subgroup of pediatric glial tumor exhibits the most aggressive vascular pathology, and affects vessels in the cortical regions and vessels located in the deeper white matter causing neuronal loss and leukoaraiosis. The vasculopathy in the IDH1mut subgroup of tumors causes mainly cortical microinfarctions. The incidence of hemorrhages is higher in the BRAFmut/FGFR2 and RTK subgroups.

**Conflict of interests.** The authors declare no conflict of interests.

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