Original Article

Vessel morphometric parameters-correlation with histologic grade and VEGF expression in oligodendroglioma

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Abstract: The contributions of histologic features including microvascular proliferation to the determination of malignancy in oligodendrogliomas remain uncertain. We have retrospectively performed morphometric assessments in 20 tumors histologically classified as well-differentiated (WHO Grade II, n=8) or anaplastic (WHO Grade III, n=12) oligodendrogliomas (WDO or AO). Quantitative studies utilized image analysis of double immunolabeled vasculature with anti CD34 with VIP chromogen (purple) and proliferating nuclei with anti MIB-1, using DAB (brown). Mean values are reported from five fields for each of twenty cases. The total number of MIB-1 positive tumor nuclei was 10 fold higher in AO vs WDO. The area occupied by vessels was also markedly increased in AO vs WDO, as was the microvessel density. Proliferating endothelial cells i.e. those with MIB-1 positive nuclei in CD34 positive cells were significantly increased (4.6 vs 0.26 positive nuclei per unit tumor area, P≤0.001) in AO. While in most areas these changes were evident as typical microvascular proliferation, other areas showed thin walled vessels with increased MIB-1 positivity. VEGF was only assessed morphologically and showed positive staining of vasculature only, in WDO, while AO also showed immunoreactivity of vessels and multiple areas of tumor cells. These findings support a contributory role for vascular proliferation in assessing histologic grade. These findings also suggest that VEGF expression which is confined to blood vessels in lower grade tumors but eventually is expressed by tumor cells in higher grade oligodendrogliomas may be an important factor as the tumor progresses.

Keywords: Oligodendroglioma, grade, vascular proliferation, VEGF

Introduction

The histologic features that have classically been recognized as important in the grading of gliomas in general, and oligodendrogliomas in particular include characteristics such as pleomorphism, mitoses, vascular proliferation, and necrosis. For a variety of reasons including the often limited sample size received for examination, fixation and processing variables, there remains a significant degree of interobserver discordance [1-3]. The presence of vascular proliferation in gliomas has always been considered an important histologic factor in tumor progression and criterion for grade since the earliest descriptions [4]. Its role in the grading of oligodendrogliomas relative to prognosis, however has often been poorly defined or shown to be statistically insignificant [5, 6]. Thus a variety of histological grading systems have attempted to grade oligodendrogliomas [7-11]. While they have recognized the significance of complex and hyperplastic vascular forms, these grading schema have not emphasized various other morphometric parameters. It is not infrequent that the histological appearance of the vasculature in a tumor may not correlate with other more malignant morphologic features present in the tumor. There has been increasing attention directed at this phenome-
non and multiple studies have now described an important predictive value to morphometric assessment of vasculature in a range of neoplasms, particularly in breast cancer. Thus increased vascular density and vascular area, as in hyperplastic vessels, may serve as markers of more aggressive behavior including the ability to metastasize, and consequently a worse prognosis. In oligodendrogliomas the relationship between classic microvascular proliferation and survival does not correlate in the same manner as it does in astrocytic neoplasms [12].

A number of factors within the tumor microenvironment have been recognized to contribute significantly to this endothelial proliferation. Vascular endothelial growth factor (VEGF) is a hypoxia induced endothelial mitogen that is well known to be upregulated in many tumors in the nervous system [13-17]. The role that VEGF may play in neangiogenesis in various tumors however continues to be defined and results of its expression in some studies on gliomas or more specifically oligodendrogliomas, have not been entirely uniform and consistent [13-17]. While this inconsistency may reflect the specific isoforms detected by different antibodies, other variables likely relate to the grade of tumors as well as the degree of vascular proliferation within neoplasms examined. With these aspects in mind, the present study sought to determine the correlation between previously defined histologic grade, morphometric parameters including vascular area, vessel density, tumor and endothelial cell proliferation as well as correlation of tumor histologic grade with VEGF protein immunoexpression.

Materials and methods

Formalin-fixed, paraffin embedded tissues of previously diagnosed cases of oligodendroglioma were selected from the surgical pathology files of the H. Lee Moffitt Cancer Center and Research Institute at the University of South Florida. Eight well-differentiated/grade II oligodendrogliomas and 12 anaplastic/grade III oligodendrogliomas were identified from consecutive cases including outside consult cases, with adequate tissue for additional studies. Hematoxylin and eosin stained sections as well as available immunohistochemistry (typically GFAP and MIB-1) and surgical pathology reports were reviewed by a neuropathologist, to confirm the diagnosis. Tissue sections were cut at 5 microns and applied to “Plus” slides (Fisher Scientific). They were deparaffinized in xylene and hydrated through graded alcohols. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol. Sections were microwaved in citrate buffer for 15 mins at low power for antigen retrieval. Following several rinses in phosphate buffered saline (PBS, pH 7.4, 0.3% Triton X-100), microvessels were highlighted with anti-human CD34 antibody (Biogenix, clone QBend/10). Primary antibody was added at 1:50 dilution and incubated for 1 hour at room temperature in a humidified chamber. Following addition of secondary antibody, the Biogenex multilink system was utilized for detection. Sections were incubated with the chromogen Very Intense Purple (VIP). Strong, dark purple membranous staining of endothelial cells was identified. Sections were double immunoreacted with the second primary, MIB-1 antibody to the nuclear cell cycle antigen Ki67, at a dilution of 1:400 also for 1 hour at room temperature in a humidified chamber. Secondary link from BioGenex (Multilink super sensitive detection kit QP900-9L) was applied. After multiple rinses in PBS, BioGenex Streptavidin was applied, incubated for 30 min and developed using diaminobenzidine (DAB) a brown chromogen, which stained proliferating nuclei. Sections were lightly counterstained with hematoxylin. Sections were dehydrated through alcohols and xylene and cover slipped with a synthetic permanent mounting medium. Positive controls included normal tonsil and negative controls included elimination of the primary antibody or use of non-specific IgG. Tumors were also separately examined for VEGF immunoreactivity using monoclonal, anti-VEGF antibody mAB293 (R&D Systems). 5 micron sections were treated with protease solution for antigen retrieval. Primary antibody was added at 1:75 dilution and sections were incubated overnight in a humidified chamber at 4°C. After multiple rinses in PBS, BioGenex Streptavidin was applied, incubated for 30 min and developed using diaminobenzidine (DAB), with a light hematoxylin counterstain.

Staining patterns were morphometrically analyzed by light microscopy and image analysis using the ImageProPlus 3D image analysis system (Media Cybernetics) in five randomly selected areas in each of the twenty tumors. Based on endothelial cell surface immunoreaction with
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CD34, each discrete vessel was identified, and the total vessel area calculated per unit tumor field. The total number of vessels per unit area of tumor was also counted. The total number of Ki67 positive (brown) tumor nuclei, as well as the total number of Ki67 positive endothelial

Figure 1. A. Histopathologic characteristics defining well-differentiated or low-grade, WHO Grade II oligodendrogliomas included diffuse, infiltrative growth pattern and presence of scattered calcification (arrow). Tumor nuclei are seen as small, round and dark, with poorly defined nucleoli and sharp nuclear membranes. Perinuclear haloes—often referred to as a “fried-egg” appearance is readily seen here. B. Additionally scattered thin-walled capillary channels, form a “chicken-wire” network. H&E stained sections low magnification. C, D. Increased cellularity, larger nuclei, prominent nucleoli with multiple mitoses (white arrows) and endothelial proliferation/hyperplasia (black arrows) H&E stained sections intermediate magnifications. E, F. Microvascular proliferation comprised of proliferating endothelial and supporting cells, forming multilayered and multilumen “glomeruloid” structures (arrows) within anaplastic oligodendroglioma.
cell nuclei (CD34 positive cell surface and MIB-1 immunoreactive nuclei) were manually tagged and counted to determine the proliferative fractions among each cell type. The vascular area per unit field, the total number of vessels per unit area, tumor proliferative fraction and endothelial cell proliferative fraction were tabulated for each of the five selected fields for each case, and then averaged for each case. The averages for histologically defined grade II, WDO and III AO tumors were compared by Student’s pooled t test. Differences were considered statistically significant if the two sided p-value was less than 0.05.

Results

Tumors were selected based on review of prior surgical pathology diagnosis and evaluation of H&E stained sections and available immunohistochemistry. The histopathologic characteristics used to define well-differentiated or low-grade, WHO Grade II oligodendrogliomas included diffuse, infiltrative growth pattern, presence of scattered calcification and neuronal satellitosis. Cytologic characteristics that are fairly typical for the oligodendroglial phenotype including features such as small, round, dark nuclei, with poorly defined nucleoli and sharp nuclear membranes. Perinuclear haloes that give these tumor cells a characteristic “fried-egg” appearance, are an artefact of fixation and not a consistent feature, although when present adds to the diagnostic characteristics. Additionally oligodendrogliomas often display a network of thin-walled capillary channels, referred to as a “chicken-wire” network. (Figure 1A, 1B). Grade III or anaplastic oligodendrogliomas display larger, more pleomor-
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<td>Tumor proliferation (# of Ki67 tumor nuclei per unit area of tumor)</td>
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Figure 3. A-C. Grade II oligodendroglioma with multiple thin-walled vessels immunoreactive for CD34 (purple) and Ki67 (brown nuclei-arrows). D-F. Grade III (anaplastic) oligodendroglioma-microvascular proliferations displaying multiple hyperplastic and multilayered vessels immunoreactive for CD34 (purple) and Ki67 (brown nuclei-arrows). F in particular illustrates that multiple thin walled vascular channels in anaplastic oligodendrogliomas are also in a proliferative stage with Ki67 positive nuclei (arrows). Double immunohistochemistry for CD34 (membranous, purple) and Ki67 (nuclei, brown). A and B: Low magnification, C: Intermediate magnification, D and E: High magnification, F: Intermediate magnification.
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Figure 4. A and B. Grade II oligodendroglioma showing variably positive tumor vessels and only rare positive tumor cells. Immunoreactivity for VEGF, hematoxylin counterstain, low and intermediate magnification. C, D. Grade III anaplastic oligodendroglioma-definite cytoplasmic immunexpression of VEGF protein in tumor cells and positive tumor vessels. Immunoreactivity for VEGF, hematoxylin counterstain, low and high magnification.

Various morphometric parameters were evaluated in all tumors. Results are expressed as mean values of 5 fields/tumor within each grade and are presented as comparisons between well-differentiated (Grade II) and anaplastic (Grade III) tumors. MIB-1 (Ki67) immunolabeling of tumors was measured to further characterize WDO vs AO. As expected, there was a significant distinction between proliferating cells in Grade II and Grade III oligodendroglioma (66.9 MIB-1 positive tumor nuclei vs 6.9/unit area of tumor, P < 0.02, Figure 2A). Mean microvessel density i.e. number of discrete, CD34 immunoreactive vessels or vascular units was significantly elevated in AO (27.9 vs 9.4/unit area of tumor, P < 0.01, Figure 2B). The area of each tumor field at 20x magnification that was occupied by vasculature was measured and compared. This was markedly increased in AO (46.3 vs 10.5/unit area of tumor, P < 0.01, Figure 2C). Finally the relationship of proliferating endothelial cells to grade

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was also examined. The number of MIB-1 immunoreactive nuclei associated with CD34 positive endothelial cells was much higher in A0 than in WDO (4.59 vs 0.26/unit area of tumor, P < 0.0001, Figure 2D). These data are summarized in Table 1.

Immunohistochemistry results for CD34 and Ki67 are illustrated in Figure 3. Low grade or well-differentiated oligodendrogioma display multiple thin-walled vascular channels that are fairly uniformly distributed throughout the tumor and are CD34 immunoreactive (purple) (Figure 3A). In addition to this, a very limited number of vessels also show positive reaction for Ki67 (brown nuclei-arrows) (Figure 3B and 3C). Anaplastic or Grade III oligodendrogioma have more complex microvascular proliferations displaying multiple hyperplastic and multilayered vessels immunoreactive for CD34 (purple) and also have multiple Ki67 positive endothelial cells (brown nuclei-arrows) (Figure 3D and 3E). An important distinction seen in some cases is that even multiple thin walled vascular channels in anaplastic oligodendrogiomas are also in a proliferative stage with Ki67 positive nuclei (Figure 3F - arrows).

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ment is the most significant factor correlating with survival.

Although the total numbers of proliferating tumor cells and endothelial cells were significantly increased in the anaplastic tumor group compared to the well-differentiated tumor group, as mentioned above, they did not correlate with classic microvascular proliferation architecture. Ki67 positive nuclear staining was commonly noted in the absence of mitoses visible by light microscopy, making the latter an uncertain marker of proliferation. While it is anticipated that vessel growth would parallel tumor proliferation, and therefore the ratio of proliferating endothelial cells to proliferating tumor cells would remain steady over the course of the tumors progression, our study shows an almost 2 fold increase in this ratio. While this increase, in our small samples, did not approach statistical significance, larger studies may indeed confirm that this ratio differs according to tumor progression, even without histologically evident vascular complexity. This would reinforce the need to consider anti-angiogenic agents in the treatment of these tumors at an earlier stage than frequently prescribed.

This investigation presents a retrospective analysis of morphometric parameters correlated with previously assigned histologic grade. It is a preliminary investigation into the importance of assessing such features, particularly in view of more recent literature on angiogenesis. Additional studies including molecular interrogation, correlation of these data with clinical features, patient survival and response to therapy are logical follow-up steps. Similarly the role that various angiogenesis-related factors may play in this process and their contribution to this increased vascular proliferative response must be further investigated.

Disclosure of conflict of interest

None.

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