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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 neoangiogenesis 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 image analysis system (Media Cybernetics) in five randomly selected areas in each of these twenty tumors. Based on endothelial cell surface immunoreaction with

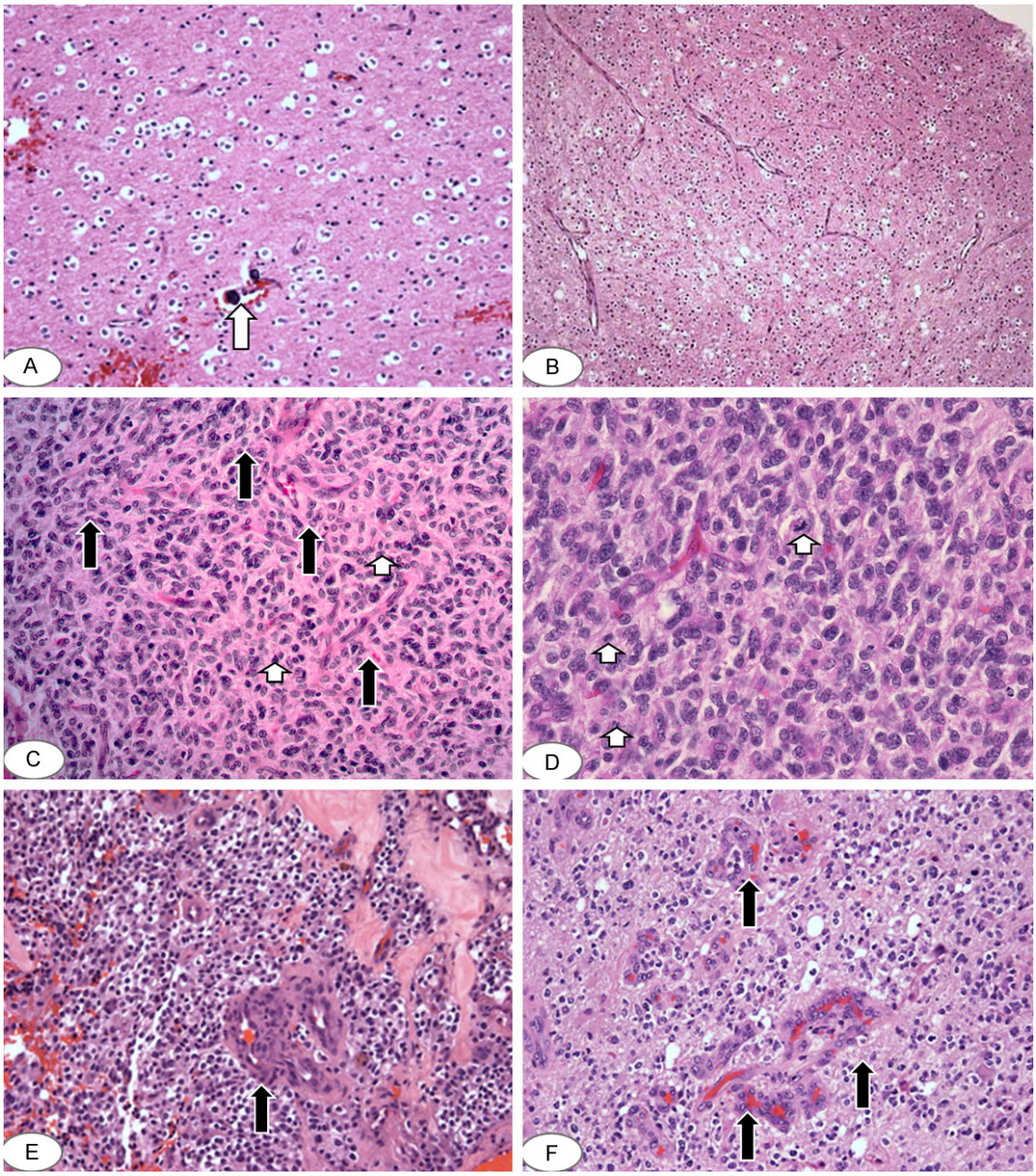

Figure 1. A. Histopathologic characteristics defining well-differentiated or low-grade, WHO Grade II oligodendrogliomas included diffuse, infiltrative growth pattern and presence of scattered calcification (arrow). Tumor nuclei are seen as small, round and dark, with poorly defined nucleoli and sharp 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.

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

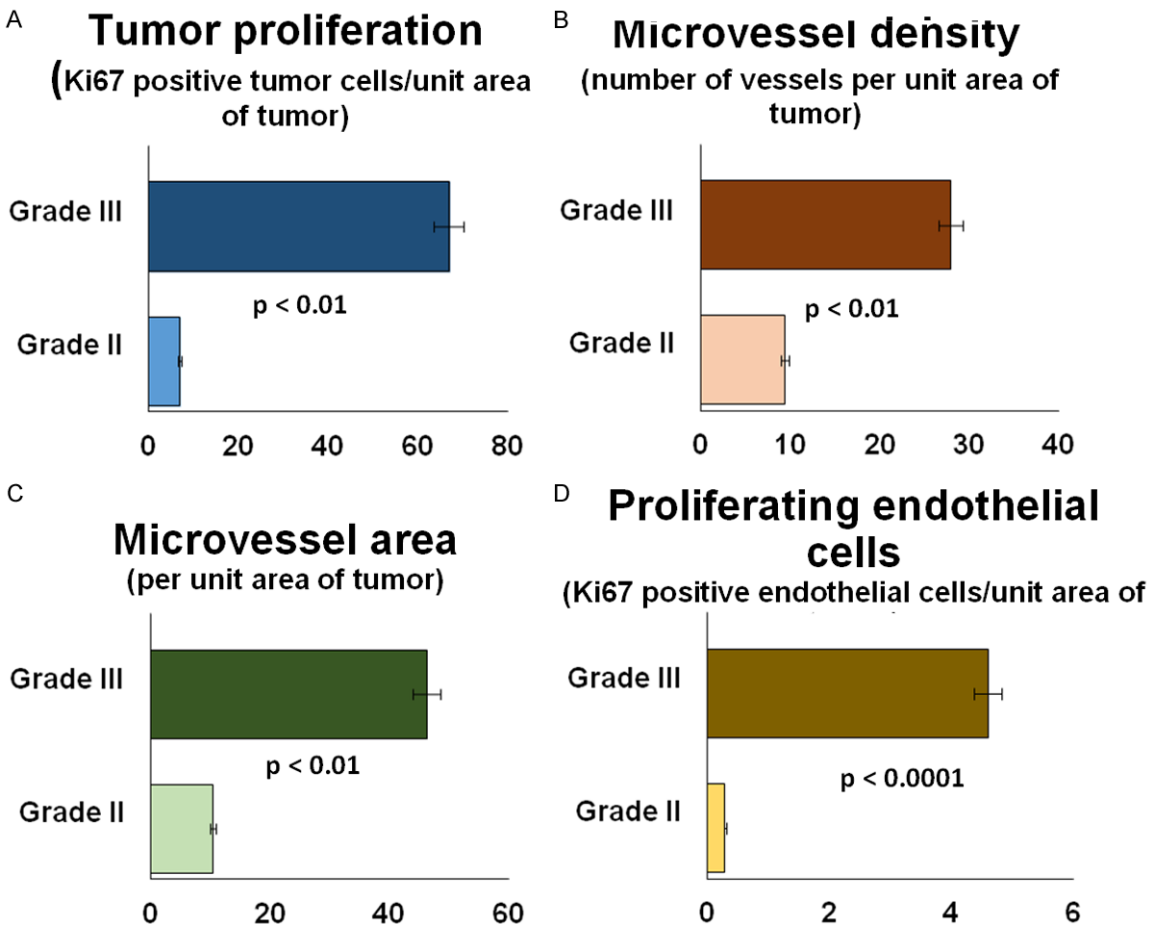

Figure 2. A. The total number of Ki67 immunoreactive nuclei were counted in 5 representative fields and are expressed as positive nuclei per unit area. B. The number of CD34 immunoreactive, discrete blood vessels in 5 representative fields, expressed as CD34 positive vessels per unit area. C. The total area of CD34 immunoreactive blood vessels measured by outlining vessels in 5 representative fields per tumor, expressed as area of CD34 positive vessels per unit area. D. The total number of MIB-1 (Ki67) immunoreactive nuclei in 5 representative fields, expressed as positive nuclei per unit area. Each bar represents the mean of the total number of Grade II or Grade III cases.

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-

Table 1. Summary of morphometric analysis

	Grade II	Grade III	*p*-value
Tumor proliferation (# of Ki67 tumor nuclei per unit area of tumor)	6.9	66.9	p≤0.02
Vascular area (per unit area of tumor)	10.45	46.27	p≤0.01
Microvessel density (per unit area of tumor)	9.4	27.89	p≤0.01
Endothelial cell proliferation (# of Ki67+ endothelial nuclei per unit area of tumor)	0.26	4.59	p≤0.001

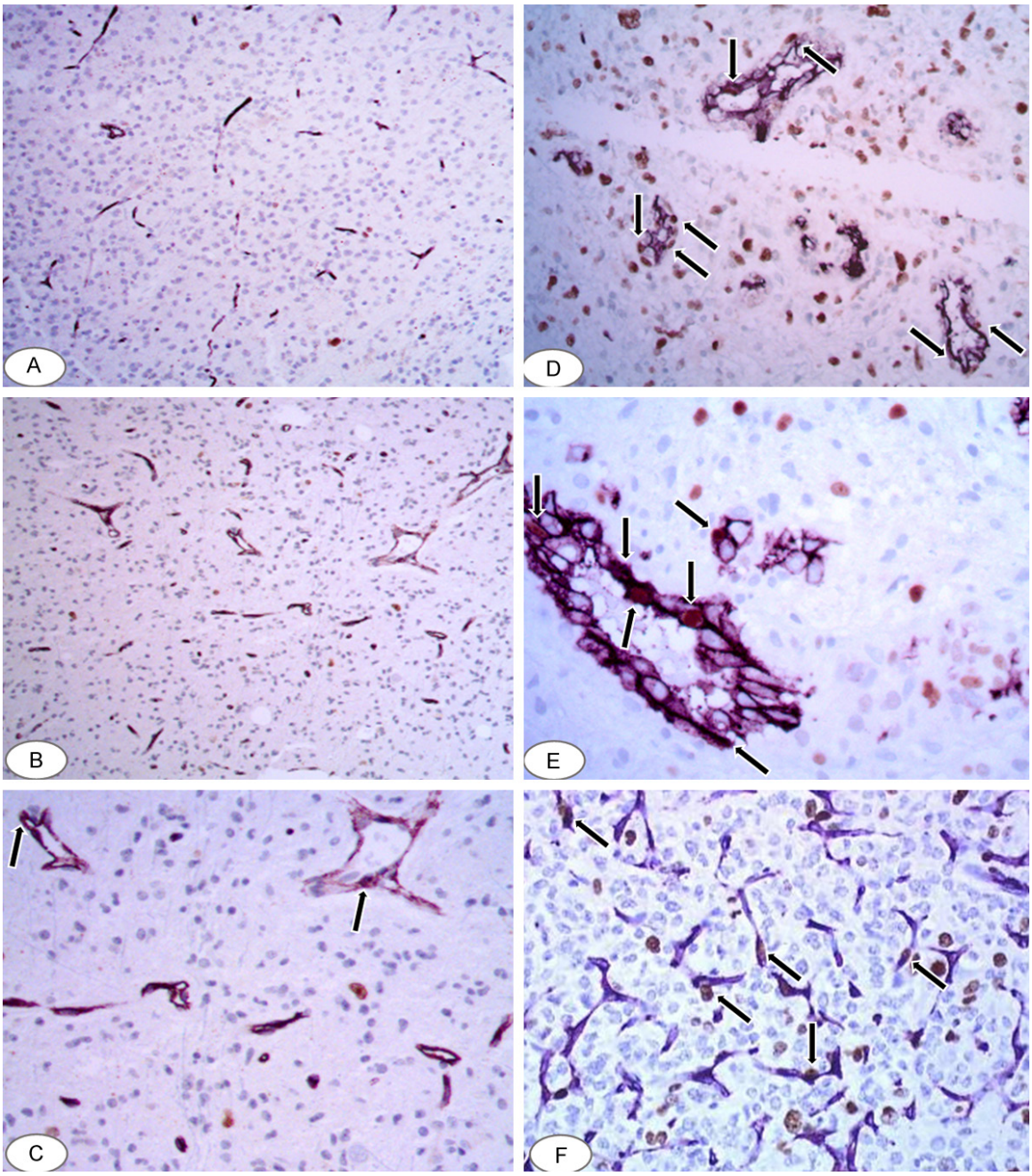

Figure 3. A-C. Grade II oligodendroglioma with multiple thin-walled vessels immunoreactive for CD34 (purple) and Ki67 (brown nuclei-arrows). D-F. Grade III (anaplastic) oligodendroglioma-microvascular proliferations displaying multiple hyperplastic and multilayered vessels immunoreactive for CD34 (purple) 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.

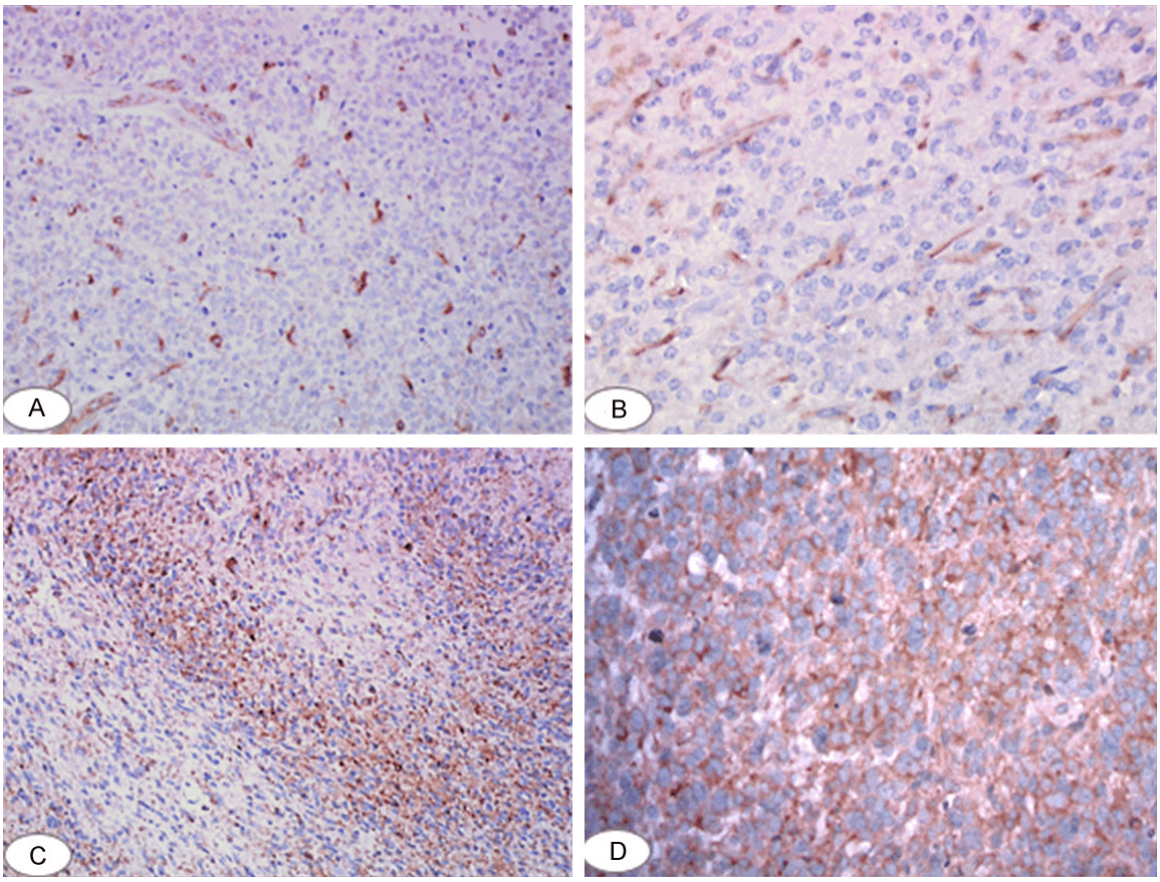

Figure 4. A and B. Grade II oligodendroglioma-showing variably positive tumor vessels and only rare positive tumor cells. Immunoreactivity for VEGF, hematoxylin counterstain, low and intermediate magnification. C, D. Grade III anaplastic oligodendroglioma-definite cytoplasmic immunoexpression of VEGF protein in tumor cells and positive tumor vessels. Immunoreactivity for VEGF, hematoxylin counterstain, low and high magnification.

phic nuclei, prominent nucleoli, frequent mitoses, scattered foci of necrosis and microvascular proliferation. The vascular hyperplasia/proliferation may retain the chicken-wire network with marked endothelial hyperplasia or may be seen as characteristic microvascular proliferation with multiple lumens and layers of endothelial and smooth muscle cells as typically seen in glioblastoma (**Figure 1C-F**). The relationship of necrosis in anaplastic oligodendrogliomas to being a predictor of poor survival does not correlate as convincingly as does necrosis in oligoastrocytomas, the latter often being classified as grade IV or glioblastoma with oligodendroglioma component [9, 18-20]. In our selection of cases we have attempted to exclude mixed gliomas-i.e. oligoastrocytoma or glioblastoma with an oligodendroglial component. Additionally our cases in general did not undergo molecular interrogation including IDH status or 1p 19q deletion analysis, as is the current common practice.

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

was also examined. The number of MIB-1 immunoreactive nuclei associated with CD34 positive endothelial cells was much higher in AO 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 oligodendroglioma 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 oligodendroglioma 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 oligodendrogliomas are also in a proliferative stage with Ki67 positive nuclei (**Figure 3F** - arrows).

Immunoexpression patterns of VEGF were fairly consistent across previously defined histologic grade of the tumors. VEGF immunostaining was confined predominantly to blood vessels only in Grade II oligodendrogliomas (**Figure 4A**, **4B**). There were only rare foci of VEGF expression in tumor cells, and in these cases also the expression was light in intensity. AO/Grade III tumors showed strong expression in many blood vessels but also prominent staining of many tumor cells in almost every case (**Figure 4C**, **4D**). We were unable to distinguish a pattern correlated with necrosis within the tumors.

Discussion

The histologic features that may contribute to the determination of malignancy within oligodendrogliomas remain uncertain. The prognostic and therapeutic implications of microvascular proliferation in many neoplasms both within and outside the CNS continue to be evaluated. Histopathologic characteristics used to define grade II vs grade III oligodendrogliomas have been fairly well defined over many years [1, 4, 8-11] and were recently reviewed [12]. The histologic grading of astrocytic tumors uses microvascular proliferation/complexity/glomeruloid architecture as a reliable criterion to distinguish WHO Grade III anaplastic astrocytomas from WHO Grade IV glioblastoma multiforme. This has also been a feature used in the grading of oligodendrogliomas Grade II (well differentiated) and Grade III (anaplastic), albeit with less reliability than in traditional astrocytic neoplasms. In this study we have morphometrically assessed the presence of vascular proliferation by combined immunohistochemical and image analysis. We determined not just the presence of classically defined microvascular proliferation but also examined the contribution of the overall number of vessels, the total area occupied by vasculature and using double immunohistochemistry also determined the proliferative fractions of tumor cells and endothelial cells.

The total number of vessels, total vessel area and average vessel area were significantly higher among the anaplastic tumors compared to the well-differentiated tumors. These numbers in multiple examples were not simply a function of microvascular proliferation. Both, the proliferating and non-proliferating vessels identified among the anaplastic as well as in well-differentiated tumors, included many thin-walled channels which often were neither complex nor glomeruloid. It is thus important to separate so called endothelial proliferation into endothelial cell hyperplasia and classic microvascular proliferation i.e complex, glomeruloid type of vascular arrangements. Daumas-Duport [11] et al proposed a new grading system for oligodendrogliomas based on morphologic and imaging criteria, with Grade A tumors lacking endothelial hyperplasia and contrast enhancement. In their sample of 79 ‘pure’ supratentorial oligodendrogliomas they correlated endothelial hyperplasia with contrast enhancement on neuroimaging. They concluded that “endothelial hyperplasia was strongly correlated with patient survival”. The median survival time between Grade A and Grade B oligodendrogliomas in their study was 3.5 versus 11 years. They also concluded that “the influence of endothelial hyperplasia on survival was similar to that of contrast enhancement”. Our morphometric data showing a very significant difference in endothelial proliferation between grades is in step with their conclusions that endothelial hyperplasia, like contrast enhance-

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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