Vascular endothelial growth factor and KIT expression in relation with microvascular density and tumor grade in supratentorial astrocytic tumors[1]

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ABSTRACT

PURPOSE: To evaluate the relationship between microvascular density and the expression of vascular endothelial growth factor (VEGF) and KIT as possible markers of angiogenic stimulus in astrocytic tumors and correlate it with histopathological grading.

METHODS: We enrolled 99 surgical specimens of supratentorial astrocytic tumors for analysis of VEGF and KIT and subsequent correlation with MVD and grading.

RESULTS: KIT and VEGF expression correlated with microvascular density ($p < 0.005$) and both VEGF and microvascular density correlated with grading ($p < 0.005$). KIT had no significant relationship with grading ($p = 0.657$).

CONCLUSION: KIT and VEGF constitute important pathways in the angiogenesis of astrocytomas and therefore are promising prognostic tools and options for therapeutic intervention.

Key words: Glioma. Neovascularization, Pathologic. Immunohistochemistry.

Introduction

Gliomas are a heterogeneous group of primary tumors in the central nervous system, and comprise 40 to 50% of all brain tumors. The most common type of gliomas are astrocytic tumors, a heterogeneous group of tumors, classified by degree of malignancy according to World Health Organization (WHO) criteria as diffuse astrocytomas (grade II), anaplastic astrocytomas (grade III) or glioblastomas (grade IV)[1]. Although glioblastomas may arise *de novo*, strong evidence indicates that some glioblastomas likely develop from low-grade astrocytomas; these tumors may also follow varying pathways of biological development[2].

All of these histopathological types can recur, and can also be phenotypically malignant, depending on the location, patient's age, health status and the extent of surgical resection. Though astrocytomas course of development can vary, the resulting outcome is frequently dismal, despite current therapeutic strategies. Astrocytomas infiltrate the brain diffusely, spreading throughout adjacent healthy tissues and recurring even after radical surgical resection[3].

Microvessel proliferation is one of the most important criteria for diagnosing malignancy in diffusely infiltrating astrocytic tumors[4]. Characterizing the molecular pathways leading to glioma angiogenesis and progression has clinical relevance because blocking molecular pathways that drive angiogenesis has major therapeutic implications[5]. Because high-grade gliomas are highly vascularized tumors, targeting blood vessels with antiangiogenic agents seems to be a promising treatment pathway[6].

Although the factors controlling glioma angiogenesis are not fully understood, experimental data support the concept that angiogenesis is required for glioma growth and that the process is driven primarily by tumor-secreted vascular endothelial growth factor (VEGF)[7,8]. The formation of primitive blood vessels from progenitors like hemangioblasts and angioblasts is dependent on VEGF, which appears to play a major role in the angiogenesis of several other human tumors, such as colon and breast carcinomas[9].

The majority of anti-angiogenic therapies already interfere with the VEGF pathway, and the results are even more encouraging when anti-VEGF antibodies are combined with chemotherapy. Trials targeting the VEGF-signaling pathways are currently under development[6].

Besides VEGF, tyrosine kinase receptors, such as KIT, are also being investigated as having an important role in the cytogenetic alterations of angiogenesis process[8]. It is known that KIT, commonly present in juvenile brain tumors, also participate in glioma angiogenesis

The proto-oncogene KIT encodes CD117, a transmembrane tyrosine kinase that has a well-established role in several human tumors; cellular expression of CD117 has already been detected by immunohistochemistry in gliomas[10]. In addition to protein expression, it has also been suggested that KIT gene amplification may be observed more commonly in glioblastomas and astrocytic tumors than in oligodendrogliomas, which constitute gliomas outside the astrocytic lineage[11]. Therefore, the KIT amplification seems to be linked to the astrocytic tumors. During angiogenesis, the KIT pathway as well as VEGF expression appears involved with astrocytoma growth, invasiveness and malignancy[11,12].

The degree of tumor vascularization can be measured by means of the microvessel density (MVD), which serves as an independent prognostic marker of survival in low-grade fibrillary astrocytomas[4,13]. It has also been suggested that tumor angiogenesis and vascular density can be used as determinants of radiosensitization, of response to anti-angiogenesis drugs and as reliable prognostic markers[13,14].

Because KIT and VEGF represent attractive possibilities for personalized therapy strategies, we sought to evaluate by immunohistochemistry the relationship between MVD, KIT and VEGF status and the correlation of these with histopathological grades in a large series of supratentorial astrocytic tumors.

Methods

This is a retrospective study based on archived slides and paraffin blocks with central nervous system tumors from the Pathology Department of the Federal University of Sao Paulo, Brazil. Local Ethics Committee approved this study (CEP 1114/07).

From 2005 to 2011, we systematically searched for all paraffin blocks of supratentorial astrocytomas that were archived before patients underwent radiotherapy. To be eligible for the study, tumors had to be identified as astrocytomas grades II, III or IV[1].

The specimens had been fixed in 10% buffered formalin and embedded in paraffin in preparation for light microscopy on hematoxilin & eosin (H&E) stained slides. Two experienced neuropathologists (TH, JNS) analyzed all H&E stained slides independently and the immunohistochemistry reactions using histopathological criteria outlined by the WHO to validate the original diagnosis, always reaching consensus.

Immunohistochemical reactions

Immunohistochemical analysis was performed using 4μm sections according to the standard streptavidin-biotin immunoperoxidase method. CD34 antigen, a transmembrane protein expressed on hematopoietic stem cells and the small vessel endothelium of various tissues, was selected to visualize the endothelial cells for MVD evaluation. The monoclonal antibody against CD34 Xm (QBEnd 10) from DAKO (Co Carpinteria, CA, USA), m7165, was used at a 1:100 dilution. The other monoclonal antibodies used included VEGF xM (C-1) from Santa Cruz (Santa Cruz, CA. USA), sc 7269, at a 1:100 dilution, and c-KIT/CD 117 xR DAKO (Co Carpinteria, CA, USA), A 4502, at a 1:100 dilution. Deparaffinized and rehydrated slides were subjected to heat-induced antigen retrieval for 15 minutes in a pressure cooker with 10 mM citrate buffer (pH 6.0) at 120 °C for CD34, or immersed in 10 mM citrate buffer (pH 6.0) during microwave heating at 700w for 15 minutes for VEGF and KIT.

Evaluation of immunohistochemistry staining

Semiquantitative scores for VEGF and KIT were applied as following. Positive immunoreactions were categorized from 0 to 3 according to the extent and intensity of staining: grade 0 for no staining; grade 1 for weak focal staining; grade 2 for strong focal staining; and grade 3 for strong widespread staining.

VEGF and KIT antigen expressions were evaluated in the cytoplasm of neoplastic astrocytes. This graduated scale was adapted from Abduldrauf *et al.*[13]. For statistical analysis purposes, 0 and 1 were classified as negative, and grades 2 and 3 were grouped as positive.

Immunostained slides for CD34 were scanned field per field at 20x to facilitate the identification of positive reactions among vascular "hot spots". Microvessel counting was performed using a 100x magnification in two different fields at the site previously defined as the "hot spots". Images of these two fields were captured with a Digital Camera System DinoCapture, and the microvessels were manually identified using the free image processing and analyzing program Image Tool 3.00. If a discrepancy occurred in the number of vessels between the assessments of the two observers, the images were reassessed for a consensual analysis. MVD was expressed, in this study, as the number of vessels counted in the two hotspots.

Slides stained for CD34 were scanned at 20x for the identification of positive reactions. Two fields considered representative of vascularity were designated as vascular "hot

spots". Images of the two hot spots were captured with a Digital Camera System DinoCapture using a 100x magnification, and the microvessels were identified and counted using the free image processing and analyzing program Image Tool 3.00. If a discrepancy occurred in the number of vessels between the assessments of the two observers, the images were reassessed for a consensual analysis. MVD was expressed, in this study, as the number of vessels counted in the two fields named as hot spots.

Statistical analysis

Statistical calculations were performed using SPSS 13.0 (SPSS Inc; Chicago, IL). In the case of a $p<0.05$, the result was considered statistically significant. Associations of MVD and KIT or VEGF expression were verified with the Mann-Whitney test; associations between MVD and tumor grade were analyzed with the Kruskal-Wallis test; and associations between tumor grade and KIT or VEGF expression were tested with Pearson chi-square tests (two-sided).

Results

The search retrieved material from 99 patients with supratentorial astrocytic tumors: 62 were men. Patients had a mean age of 46 years (range 20-78 years). Among the 99 patients, 58 (58.6%) had diffuse astrocytomas (WHO grade II); 25 (25.3%) had anaplastic astrocytomas (WHO grade III); and 16 (16.2%) of the patients had glioblastomas (WHO grade IV).

VEGF expression in grades II and III astrocytomas and in glioblastomas is described in Table 1.

TABLE 1 - Mean microvascular density (MVD) and assessment of vascular endothelial growth factor (VEGF) and KIT positive immunohistochemical reactions distributed according to tumor grade.

Grade by WHO	MVD (median; range)	VEGF positivity	KIT positivity
II	24 (13-44)	25 (43.1%)	15 (25.9%)
III	33 (22-38)	16 (64%)	7 (28%)
IV	35 (28-49)	12 (75%)	6 (37.5%)
Total	29 (13-49)	53 (53.5%)	28 (28.3%)

WHO=World Health Organization[1]

KIT expression was detailed in the same Table. In the vast majority of cases the expression for both markers, when

present, was diffuse.

Correlation of MVD with KIT and VEGF expression

The MVD was significantly higher for the group of tumors that expressed VEGF (p<0.005). The median MVD of the positive group was 34 (range 21-49), whereas the negative-for-VEGF group had a median of 23 (range 13-35), as illustrated in Figure 1.
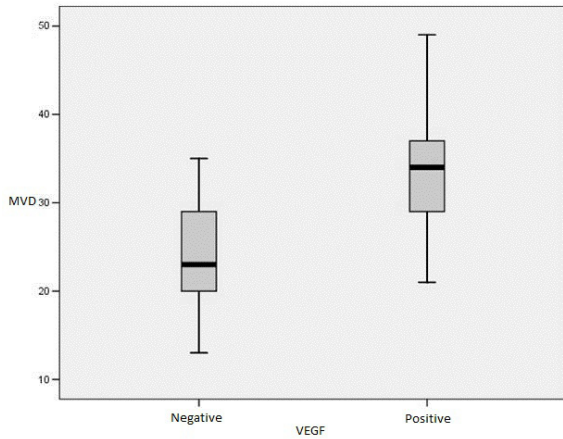

FIGURE 1 - Correlation between microvessel density (MVD) and vascular endothelial growth factor (VEGF) expression (Mann-Whitney test).

MVD was also significantly higher for the group of tumors that expressed KIT (p<0.005). The median MVD of the positive group was 34 (range 27-49), whereas the negative for the KIT group had a median of 25 (range 13-39), as shown in Figure 2.
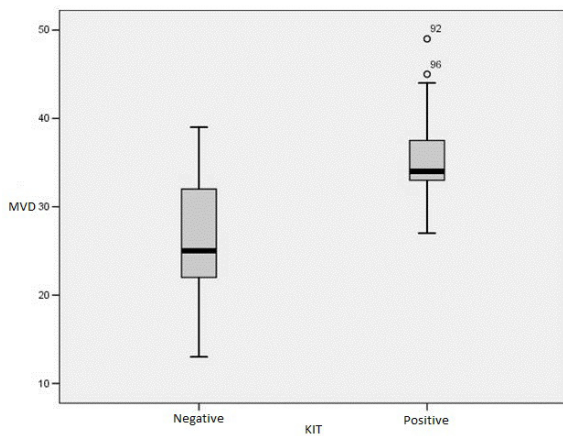

FIGURE 2 - Correlation between microvessel density (MVD) and KIT expression (Mann-Whitney test).

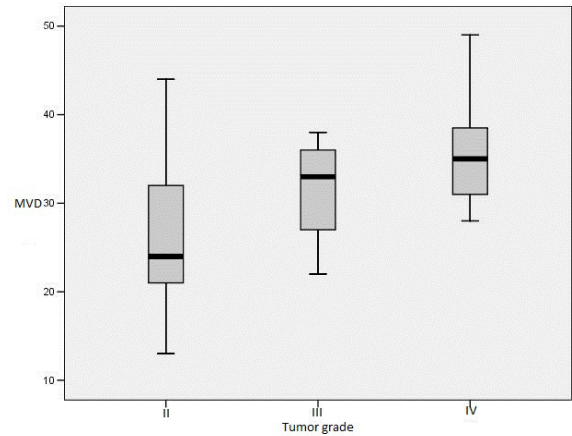Tumor MVD was significantly associated with tumor grading (p<0.005), as shown in Figure 3.

FIGURE 3 - Correlation between microvessel density (MVD) and tumor grade (Kruskal Wallis test).

Correlation of tumor grading and VEGF and KIT expression

The grading of gliomas correlated significantly with VEGF expression (p<0.037). There was no association between KIT expression and tumor grade (p=0.657). Images of KIT and VEGF expression as well as microvascular density with CD34 staining are shown in Figure 4.

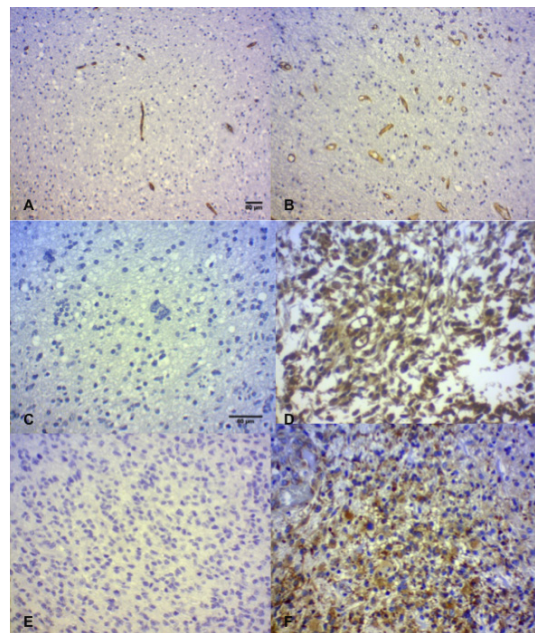**FIGURE 4** - **A**. Low microvessel counting in a diffuse astrocytoma (grade II). **B**. High microvessel counting in an anaplastic astrocytoma (grade III). **C**. Vascular endothelial growth factor (VEGF) expression absent in an anaplastic astrocytoma (grade III). **D**. VEGF expression positive in a glioblastoma (grade IV). **E**. KIT expression absent in an anaplastic astrocytoma (grade III). **F**. KIT expression positive in an anaplastic astrocytoma (grade III). (A, B: CD34 staining, original magnification 100x; C, D: VEGF staining, original magnification 200x; E, F: KIT staining, original magnification 200x).

Discussion

Low-grade astrocytomas constitute a minor group of all astrocytic tumors and have an inherent tendency to become higher grade tumors through malignant transformation. Despite some well-known factors related to prognosis, no unambiguous marker can predict astrocytoma progression[15]. Astrocytomas are a heterogeneous group of gliomas, and accurate histopathological diagnosis is crucial to define the prognosis of these tumors, which differ primarily in their abilities to grow and progress[1].

The malignant astrocytoma variants (grades III and IV) are the most frequently diagnosed primary brain tumors in adults. The migratory behavior of these malignant cells limits the effectiveness of surgical treatment, and the life expectancy of patients with glioblastomas, using current standard care, is an average of 14 months after diagnosis, despite the type of surgery, radiation and chemotherapies employed[3].

Tumor microenvironments and genetic abnormalities that drive glioma growth, progression to malignancy and resistance to therapy are critical to optimize prognostic accuracy and to design optimal therapeutic strategies. Angiogenesis is fundamental for human reproduction and for wound healing, but it is also crucial for tumor progression, growth and metastasis. Angiogenesis is often present in high-grade lesions, and can be used as a prognostic factor. Many studies have focused on these characteristics and associations[16,17].

Leon *et al.*[16] reported rich angiogenic activity in gliomas. Brain tumor growth was shown to depend largely on blood supply to provide nutrients and to remove necrotic debris from the central area of the neoplasm; a reciprocal relationship exists between the hemostatic system and tumor growth, cellular differentiation and metastasis. MVD is the final step of the whole process, and research dedicated to it allowed many authors to correlate certain tumors' biological aggressiveness with vascular proliferation[17]. In our study, we found a strong relationship between vascular proliferation and tumor grade (p<0.005), which reinforces the importance of MVD status and glioma classification and prognosis because malignant astrocytoma progression is heralded and accompanied by increased angiogenesis[18].

Usually, molecular control of the angiogenic process entails regulating multiple steps that involve the recruitment and proliferation of endothelial cells, mediated by proangiogenic factors. Although there are many known angiogenic factors, vascular endothelial growth factor (VEGF) is certainly the major regulator in the case of gliomas and many other tumors[13]. Some reports have described the positive correlation of VEGF, vascular

density and perfusion-weighted-imaging in gliomas[19]. The findings reported herein support this correlation because we found a relevant positive relation between VEGF expression and MVD (p<0.005). VEGF expression was also found to be significantly related to tumor grade (p=0.037). Yoo *et al.*[20] reported similar findings in a series of 78 WHO grade II, III and IV astrocytic tumors. The cytokine VEGF is a multifunctional, endothelial cell-specific mitogen and angiogenesis inducer *in vivo*, and, currently, a number of clinical trials targeting the VEGF-signaling pathways are being developed[6]. Bevacizumab, a humanized anti-VEGF antibody has shown promising results in exploratory phase II trials of recurrent GBM[21]. Unfortunately, the response to anti-VEGF therapy is usually transient, and the majority of patients eventually relapsed, so new data is needed to understand the mechanisms of tumor escape[22].

Another interesting therapeutic option is the novel targeted agent for KIT. Stem cell factor (SCF) is the ligand of the KIT receptor tyrosine kinase, which is codified by the gene KIT, located on chromosome 4q12 along with two other receptors named PDGFRA and VEGFR2, all of which are frequently amplified in primary glioblastomas[12]. SCF is up-regulated in high-grade gliomas; it promotes angiogenesis and the survival of central nervous cells and is associated with a short survival[23]. Because those receptors are preferentially expressed in tumor cells, their inhibition poses an attractive therapeutic option that might result in far fewer side effects than that observed in standard treatments.

SCF/KIT signaling pathways are involved with nervous system development, and interact with signal transduction pathways involved in carcinogenesis and cellular differentiation that regulate many crucial steps of tumorigenesis; their activation is involved with angiogenesis and gametogenesis[24]. Stimulation of KIT with SCF results in dimerization, autophsphorylation and the resultant activation of downstream effector proteins that have already been widely investigated by neuroscientists because the KIT gene is highly amplified in both low and high-grade gliomas[25]. Primary human gliomas express KIT and induce normal neurons to express KIT in brain regions infiltrated by glioma cells when these areas present prominent angiogenesis[26].

Sun and co-workers validated the hypothesis that KIT activation plays a fundamental role in glioma angiogenesis[27]. Its activation may promote these tumors' peculiar angiogenic patterns, which relate to gliomas' histopathological subtypes and vascular proliferation[28]. Our findings support this hypothesis because we found a positive correlation between KIT expression and MVD (p<0.005). Nevertheless, no correlation was found between the expression for KIT and the tumor grade (p=0.657). These findings

are consistent with those of Gomes *et al.*[11], in that out of a series of 179 gliomas of various grades, a positive expression for KIT was found in 25% (1/4) of pilocytic astrocytomas, 25% (5/20) of diffuse astrocytomas, 20% (1/5) of anaplastic astrocytomas and 19.5% (15/77) of glioblastomas and no association was found between KIT tumoral overexpression and patient survival. Saini *et al.*[29] also found that, contrary to earlier reports, KIT expression was seen in 32.5% (13/40) glioma cases with no association to tumor grade. Those findings of KIT over-expression across low/high grades in glioma cases are similar to ours and suggest that it may not be a molecular abnormality accumulated during tumor progression. Therefore, it is essential to elucidate whether the enhanced KIT expression is a cause or outcome of the glial neoplastic process[29]. Conversely, conflicting reports suggest that tumor aggressiveness depends on an increased expression of KIT and its activated form[24]. Cetin *et al.*[10] studied a series of 52 glial tumors and found that 75% were positive for KIT, and the proportion of high-grade tumors was significantly greater than of low-grade tumors.

Conclusion

The vascular endothelial growth factor and KIT are markers associated with microvessel density and both VEGF and MVD correlated with grading. KIT had no significant relationship with grading in astrocytic tumors.

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