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Evaluation of the microvascular density in astrocytomas in adults correlated using SPECT-MIBI

SANDRO PANTOJA CAVALCANTE[1,5], JOSÉ REYNALDO WALTHER DE ALMEIDA[1], CARLOS AFONSO CLARA[1], CRISTOVAM SCAPULATEMPO NETO[2], STELA VERZINHASE PERES[4], SÔNIA MARTA MORIGUCHI[3], MARCELO JOSÉ DOS SANTOS[3] and EUCLIDES TIMÓTEO DA ROCHA[3,5]

[1]Neuro-oncology Division, Department of Surgery; Departments of [2]Pathology, and [3]Nuclear Medicine; [4]Research Support Group, Hospital de Câncer - Fundação Pio XII, 14784-400 Barretos; [5]Blood Transfusion Center, Medical School, São Paulo State University (UNESP), 18618-000 Botucatu, São Paulo, Brazil

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Abstract. Microvascular density (MVD) may be an additional prognostic marker for astrocytomas, but the heterogeneity of these tumors limits its use. Thus, imaging examinations such as SPECT-MIBI (2-methoxyisobutyl isonitrile) may take on an indirect role in astrocytoma evaluation. The aim of this study was to evaluate MVD in astrocytomas using immunohistochemistry with anti-CD34 monoclonal antibodies. The relationship between the immunohistochemical data and the parameters obtained from SPECT-MIBI was evaluated. This cross-sectional study evaluated 48 patients with brain tumors including low-grade astrocytomas (LGAs), anaplastic astrocytomas (AAs) and glioblastoma multiformes (GBMs). Patients had been admitted to the Hospital de Câncer de Barretos - Fundação Pio XII, and underwent brain SPECT-MIBI prior to any treatment. MVD was determined under an optical microscope by counting microvessels on slides from each case. SPECT-MIBI images were analyzed visually and semiquantitatively. GBMs, AAs and LGAs represented 50, 16.7 and 33.3% of the total sample, respectively. There were 13 normal and 35 abnormal SPECT-MIBI images. Significant differences in MVD were found between AA and LGA cases (p=0.040), but not between normal and abnormal SPECT-MIBI. The mean counts from SPECT-MIBI were not correlated with MVD. Among the GBM cases, there were no significant findings, except for an increased likelihood of abnormal histological test results. MVD was related to histological grade (in AA and LGA cases) but was not correlated with SPECT-MIBI.

Correspondence to: Dr Sandro Pantoja Cavalcante, Hospital de Câncer - Fundação Pio XII, Avenida Antenor Duarte Vilela, 1331, Bairro Dr Paulo Prata, 14784-400 Barretos, São Paulo, Brazil
E-mail: sand_pc@hotmail.com

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Introduction

Among the tumors that affect the brain, gliomas of astrocytic, oligodendroglial and ependymal origin are responsible for more than 70% of tumor occurrences (1). Of these, astrocytomas are the most frequent histological type. They are classified according to their malignant potential and graded on the basis of histological criteria recommended by the World Health Organization (WHO) (2). Over the last few years, knowledge of the mechanisms of tumor progression leading to astrocytomas has increased. This has led to the supplementation of histological grade with new potential prognostic markers. Among these, there has been increasing interest concerning the role of the quantification of vessels in astrocytomas, with ongoing investigations being conducted (3).

In 1991, Weidner *et al* were the first to report on the prognostic value of measurements of angiogenesis using a model of primary breast tumors (4). Many investigators have supported the notion of an inverse correlation between microvascular density (MVD) and prognosis (5-7), while others have refuted this (8). MVD has also been shown to be a prognostic indicator of postoperative survival among patients with astrocytomas (3,9,10). However, the quantification of the vascularization of brain tumors has been limited due to the cell heterogeneity found in these tumors (9). Other authors have demonstrated significant differences in MVD between tumors in children and adults (3).

For brain tumors, MIBI (2-methoxyisobutyl isonitrile) has been shown to be accurate in identifying recurrence following treatment for high-grade gliomas, thereby making it possible to differentiate them from radionecrosis (11), although there are divergent opinions on this subject (12). Furthermore, MIBI aids in differentiating between the findings from neoplastic and non-neoplastic lesions (11,13), allows tumor grade to be evaluated (11), and assists in predicting therapeutic response (11,14,15). The intensity of uptake is related not only to breaking the blood-brain barrier, but also to tumor metabolic activity (11,16,17). Thus, MIBI may be used to evaluate the biological characteristics of brain tumors and helps to determine their proliferative potential and prognosis (11,18,19).

Because of its molecular characteristics, MIBI is preferentially absent in mitochondria (20,21). Since the mitochondrial activity in tumors is usually high, this generates an increase in the transmembrane gradient, which favors its accumulation in tumor tissue significantly.

Vascularization is important as a prognostic factor in many types of neoplasias (3-5,22,23), but such evaluations are only possible today through direct analysis of slides using several methods (4,22) that all have limitations due to the heterogeneity of these tumors (3). Various imaging techniques have been proposed as valuable tools for evaluating vascularization in brain tumors, among them SPECT-MIBI (16,17). This method has revealed a significant correlation between MIBI uptake rates and length of survival, according to the aggressiveness of the recurrent malignant gliomas (24). However, the amount of robust information on its relationship with MVD remains insufficient, thus allowing for new perspectives regarding this approach.

The aim of this study was to evaluate MVD in low-grade astrocytomas (LGAs), anaplastic astrocytomas (AAs) and multiform glioblastomas (GBMs) using immunohistochemistry with anti-CD34 monoclonal antibodies to determine the relationship between immunohistochemical data and the parameters obtained from SPECT-MIBI.

Materials and methods

Study population and protocol. A study of cross-sectional type was conducted, within which demographic and KPS (Karnofsky performance scale) data were gathered retrospectively from 48 patients (29 men and 19 women; mean age 48.8 years, SD 15.9, range 20-73 years) who had been admitted to the Hospital de Câncer de Barretos - Fundação Pio XII. None of these patients had undergone any previous surgical or therapeutic procedures. Only cases of supratentorial gliomas that had been diagnosed in accordance with WHO criteria (2) as LGAs (of which only diffuse astrocytomas were selected), AAs or GBMs were included. All patients underwent brain SPECT with MIBI before any procedures. Tumor tissue specimens of an adequate quantity were available in paraffin blocks for all patients. The study was approved by the ethics committee of the Hospital de Câncer - Fundação Pio XII.

Acquisition of brain SPECT images with MIBI. Examinations were performed 15 min after intravenous administration of 720 MBq of MIBI labeled with Tc99m. Tomographic images were captured using a GE Millenium VG gamma camera equipped with two high resolution detectors, with a 128x128 matrix and 360˚ rotation, thus making it possible to obtain 120 two-dimensional frames of the brain, 25 sec/frame. The images obtained were reconstructed in a 128x128 matrix using Butterworth-filtered back-projection, with a cutoff of 0.25 and order of five (25), in accordance with a semi-automated protocol furnished by the manufacturer. The examinations were interpreted by an experienced nuclear medicine physician in the Department of Nuclear Medicine of Hospital de Câncer - Fundação Pio XII.

Interpretations were initially performed by visual analysis and classified as normal or abnormal. The latter were then subdivided into abnormal images of mild intensity (when uptake could be discerned, but less than in the scalp), moderate intensity (when the uptake was as intense as the scalp) and marked intensity (when the uptake was equivalent to the salivary glands). Semiquantitative analysis was performed to investigate the mean count in the tumor area and in a mirror area in the contralateral hemisphere (14,16,24,26). This made it possible to obtain an index (tumor/contralateral side index, T/CL) as the ratio between these measurements.

Table I. Description of the clinical, surgical and anatomopathological data and visual analysis of the SPECT-MIBI results.

Variable	n	%
Symptoms or signs of presentation		
Headache	18	37.5
Convulsions	7	14.6
Motor deficit	12	25.0
Associated and other symptoms	10	21.0
Unknown	1	2.1
KPS (%)		
≤40	1	2.1
50-60	8	16.7
70-80	19	39.6
90-100	9	18.8
Unknown	11	22.9
Intervention		
Biopsy	12	25.0
Surgery	36	75.0
Anatomopathology		
Glioblastoma multiforme	24	50.0
Anaplastic astrocytoma	8	16.7
Low-grade astrocytoma	16	33.3
Visual analysis		
Normal	13	27.1
Mildly abnormal	10	20.8
Moderately abnormal	18	37.5
Markedly abnormal	7	14.6
Total	48	100.0

KPS, Karnofsky performance scale.

Histopathological classification. Slides stained with H&E were produced from the paraffin blocks in order to review the diagnoses and the histopathological grade. These reviews were performed by two experienced pathologists following WHO criteria (2).

Immunohistochemical reactions. Sections (3 μm) were cut from the paraffin blocks from each case. These were mounted on slides and silanized (3-aminopropyltriethoxilane, A-3648, Sigma, USA) and deparaffinized in a heated chamber at 60˚C for 12 h for immunohistochemical reactions.

Table II. Description of the mean count parameters and MVD.

Variable	No. of examinations	Mean (SD)	Median
MCT	35	1226.1 (1132.3)	817.0
MCMI	35	285.2 (291.1)	185.0
T/CL index	35	5.8 (5.4)	3.9
MVD	48	71.1 (33.9)	64.9

MCT, mean count in the tumor; MCMI, mean count in the mirror image; T/CL, ratio of mean counts in the tumor and on the contralateral side; MVD, microvascular density.

The streptavidin-biotin-peroxidase technique was used with anti-CD34 class II monoclonal antibodies (clone QBEnd-10, code m7165, titration 1:800; Dako Cytomation, Glostrup, Denmark). Antigen recovery was carried out on a Pascal pan, using citrate at pH 6.0. Amplification was performed using the Advance™ HRP System (Dako Cytomation).

Microscopic analysis of immunohistochemical reactions. MVD was analyzed as described by Weidner *et al*, with some modifications (4). Two observers without knowledge of the case diagnoses viewed the slides under a dual-view optical microscope (Olympus BX 41) at a magnification of x40, searching for the areas of greatest concentration of immunolabeled vessels (hot spots). At each hot spot, a magnification of x200 was used to photograph the optical field. A total of three fields were photographed, one in each hot spot of each tumor. Adobe® Photoshop 7.0 software was used to open and amplify the images. The vessels were counted on the computer screen. Individual microvessels (arterioles and venules) were counted in each of these areas, and the MVD was determined as the ratio of the total number of microvessels counted divided by the three fields examined (17).

Any CD34-positive endothelial cells or groupings of endothelial cells (clusters) that were clearly separated from the tumor cells and other glial elements were considered to be a single countable microvessel. Those that appeared to be derived from the same vessel, if separate, were also counted. Each fixed lumen was counted as a single countable microvessel. If there was no lumen, but only a single CD34-positive cell visible, this cell was also interpreted as a single microvessel (3,4,17).

Statistical analysis. A descriptive analysis was performed on the data in terms of frequencies and percentages, central trend and dispersion measurements, means, medians, standard deviations, minimums and maximums. The Kolmogorov-Smirnov test was applied to investigate the adherence of the quantitative variables to a normal distribution curve. For the dependent variable of MVD, this test was applied in accordance with the subdivisions of each demographic, clinical and interventional category (27). For associations between qualitative variables relating to anatomopathology and visual analysis, the Fisher's exact test was applied.

Since some variables did not present a normal distribution, it was decided to use non-parametric tests. To compare the means between the independent variables and MVD, the Mann-Whitney test was applied when the independent variable had two categories, and the Kruskal-Wallis test was used when the independent variable had more than two categories. To investigate correlations between the dependent variable (MVD) and the tumor/contralateral side (T/CL) index, Spearman's correlation was used. Statistical significance was set as $p<0.05$. Data input, consistency and analysis were performed using SPSS software for Windows, version 16.0.

Results

As shown in Table I, the single forms of clinical presentation most commonly found were headache (37.5%), motor deficit (25%) and convulsions (14.6%). Most of these patients (81.3%) presented KPS of ≥70%. Among the paraffin-block specimens, 75% were obtained from surgical procedures with full or partial macroscopic resection of the tumor, and the remainder came from biopsies. GBMs accounted for 24 cases (50%) of the sample, while the LGA and AA groups represented 16 (33.3%) and 8 cases (16.7%), respectively. Visual analysis of the SPECT-MIBI examinations did not find any abnormality in 13 examinations (27.1%), while the remaining 35 examinations (72.9%) presented mild, moderate or marked abnormalities.

Among these 35 abnormal examinations, the mean count in the tumor was 1226.1, and in the mirror image it was 285.2. The mean T/CL index was 5.8. The mean number of vessels found in the 48 slides was 71 vessels/field as shown in Table II.

In analyzing the differences in mean MVD in relation to gender and the interventions that gave rise to the specimens, there was no significance except in relation to the anatomopathological variable ($p=0.013$). However, the MVD for the GBMs was lower, with a mean of 58.8 vessels/field, while for the AA and LGA cases it was 107.4 and 71.4 vessels/field, respectively, as indicated in Table III.

The difference in mean MVD between the AAs and LGAs was significant ($p=0.040$). However, there was no significant difference in mean MVD between normal and abnormal examinations ($p=0.057$), as shown in Table IV. A similar analysis for the GBM group did not reach significance ($p=0.295$) (result not shown in the Table). Correlation between the T/CL index and MVD was also non-significant, as shown in Table V.

On the other hand, Table VI indicates that there was a greater likelihood that the GBM group would present abnormal examination results in relation to the AA and LGA cases (87.5 vs. 58.3%; $p=0.049$).

Table III. Difference in the mean MVD between the categories.

Variable	No.	Mean (SD)	Median	p-value
Gender				
Male	29	70.7 (33.9)	71.3	0.983[a]
Female	19	71.8 (34.9)	63.3	
Intervention				
Biopsy	12	69.4 (41.1)	51.1	0.668[a]
Surgery	36	71.7 (31.7)	68.9	
Anatomopathology				
Glioblastoma multiforme	24	58.8 (20.0)	55.9	0.013[b]
Anaplastic astrocytoma	8	107.4 (42.4)	114.1	
Low-grade astrocytoma	16	71.4 (34.8)	76.0	

[a]Mann-Whitney test; [b]Kruskal-Wallis test.

Table IV. Difference in the mean MVD between anaplastic astrocytomas and low-grade astrocytomas, and between examinations with normal and abnormal uptake.

Variable	No. of examinations	Mean (SD)	p-value
Anatomopathology			
Anaplastic astrocytoma	8	107.4 (42.40)	0.040[a]
Low-grade astrocytoma	16	71.4 (34.82)	
Uptake			
Normal	10	64.9 (34.84)	0.057[a]
Abnormal	14	96.6 (40.13)	

[a]Mann-Whitney test.

Table V. Correlations of quantitative variables with MVD.

Variable	No.	r-value	p-value
T/CL index in LGA and AA	14	0.238	0.413[a]
T/CL index in GBM	21	-0.084	0.716[a]

T/CL, ratio of mean counts in the tumor and on the contralateral side; LGA, low-grade astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme. [a]Spearman test.

Discussion

In the present study, data on 48 adult patients with supratentorial astrocytomas were evaluated in an attempt to establish a relationship between MVD in these tumors and the results of analysis using SPECT-MIBI. Gender did not influence MVD, which has not been previously discussed in the literature. GBMs accounted for 50% of the sample evaluated as shown in Table I, although there was no randomization. Despite the lower diagnostic accuracy provided by biopsies (28), which suggests that studies with such samples should be regarded separately (9), in our study there was no significant difference in mean MVD between the samples obtained through biopsy and those obtained through surgery, as shown in Table III.

There have been discussions on the relationship between MVD and tumor prognosis, not only outside the central nervous system (5,7,23), but also within (3,9,10). Moreover, the presence of vascular proliferation determined non-quantitatively distinguishes high- and low-grade astrocytomas (2). For this reason, it would be expected that a greater MVD would be obtained with increasing tumor grade. Such a relationship was found between the LGAs and AAs, but not for the GBMs, as indicated in Table III. In 2005, Yao *et al* found a significant difference only between GBMs and LGAs (10), while Leon *et al,* considering only AA and GBM cases in 1996, showed that there was a significant association between microvascular grade and histology (9). Furthermore, the latter author showed that microvascular grade and MVD were significant predictors of postoperative survival, and that the vascular grade was more intense, which was explained by the existence of glomeruloid vascular structures. In fact, these structures are clusters of vessels that influence the choice of hotspots, given that the microvascular grade is higher in these areas. Nonetheless, they are counted as single vessels

Table VI. Likelihood of abnormal examination result in relation to histological type.

Visual analysis	Anatomopathology		
	LGA/AA	GBM	
	No. (%)	No. (%)	p-value[a]
Normal	10 (41.7)	3 (12.5)	0.049
Abnormal	14 (58.3)	21 (87.5)	
Total	24 (100.0)	24 (100.0)	

LGA, low-grade astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme. [a]Fisher's exact test.

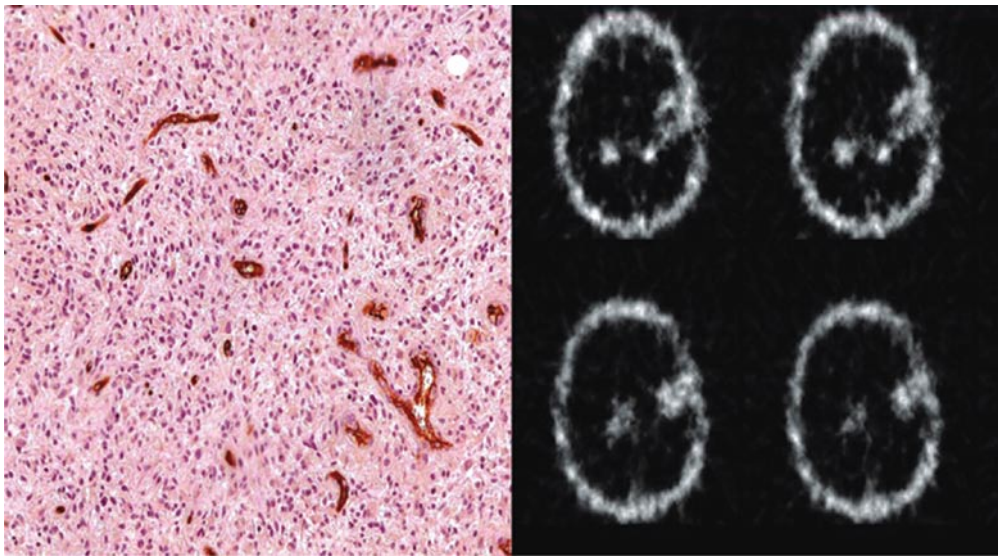

Figure 1. Glioblastoma multiforme with few vessels and marked uptake.

at a higher magnification, thereby probably resulting in an underestimation of the count of individual microvessels (9). Extensive areas of necrosis also confer additional explanations, although, in the present study, there was constant attention to avoiding areas that were close to necrosis. The existence of necrosis makes immunohistochemical analyses difficult (29), while MVD has failed to show any correlation with histological grade in tumors presenting necrosis (30). Tumor necrosis, and in particular large-scale ischemic necrosis, is significantly more frequent in primary GBMs (31,32). Therefore, the presence of glomeruloid formations and necrosis, which were elements observed in the present study, may explain why there was an underestimation of the microvessel counts in the GBMs. It was understood that the identification of hot spots, from the manner in which this was carried out, might not faithfully represent the appropriate area for counting the vessels inside the tumor. This would particularly be the case in GBMs, in which the heterogeneous characteristic of brain tumors appeared to be more evident.

This heterogeneity has limited the use of pathological analysis techniques (9), thus explaining why no relationship between tumor grade and MVD was found by some authors (33). Moreover, different methods have been used to quantify MVD (17,34), which leads to a lack of standardization and additional difficulties in establishing the real role of vessel quantification as a prognostic indicator.

It is known that 95% of GBMs start out as GBMs, while only 5% originate from LGAs or AAs. Thus, GBMs may be primary or secondary. These constitute distinct entities that affect patients at different ages and develop through different genetic routes. LGA and AA represent a continuum within the disease, with similar genetic characteristics, and thus differ from GBM, which has separate genetic characteristics (35,36). In the present study, out of the 48 specimens evaluated, there were 24 GBMs, which were studied separately. It is important to note that, due to the presence of extensive areas of necrosis, it was not possible to establish a correlation between MVD and the GBMs.

Anti-CD34 monoclonal antibodies were chosen for the present study. Although they are non-specific for endothelial cells, they present good sensitivity for vascular endothelium (37) and are an efficient vascular marker for glial tumors (33). Several other markers have been described, but none have been indicated as the most recommended or most efficient. Comparative studies on different antibodies have shown divergent results, particularly with regard to prognosis

(7,23,33). The main criticism that could be directed towards anti-CD34 is that it not only marks the neoformed vessels, but also the normal vessels existing inside the tumor (5,7). Furthermore, it has been demonstrated that not all neoformed vessels have evident circulation (17). It should be emphasized that this peculiar characteristic of anti-CD34 was regarded as a point of interest by the present authors, since they judged that the SPECT-MIBI images would be dependent on the vascular supply of the tumor.

The mechanisms for MIBI uptake are still not well defined, although some authors have reported that MIBI uptake does not depend on MVD and only reflects the rupturing of the blood-brain barrier (16,17). Nonetheless, the existence of methodological inconsistencies in quantifying MVD means that its true value in relation to MIBI uptake cannot be assessed with certainty. MIBI has been widely used to differentiate radionecrosis from tumor recurrence (11), but there were three cases of GBM in the present study in which no abnormal areas of uptake were viewed (Table VI). A similar finding was described previously (12,17), which indicates the need for caution in interpreting these examinations.

There was a significant difference in mean MVD between LGA and AA cases, but a significant difference (p=0.057) was not noted between uptake and MVD (Table IV). However, we believe that the sample size may have influenced this result, and that evaluation of a larger sample would lead to the finding of significance in this tumor group. In 2004, Staudenherz *et al* stated that MVD is not crucial for the scintigraphic viewing of brain tumors using 99mTc-MIBI (17). In their sample, there were four cases, of which three were astrocytomas and one was an oligodendroglioma. There were no associations between histological grade and MVD, and only a descriptive analysis was made.

The T/CL indices obtained in the present study did not present any significant correlation and, in addition, the index was negative for the GBMs (Table V and Fig. 1). In addition, there was no significant difference between the means of the examinations with normal and abnormal uptake among the GBM cases. There have been many studies on MVD or SPECT-MIBI in brain tumors (3,9,10,12,14,16-19,24-26,33,34,38), but this is the first that sought to establish a relationship between MVD and a numerical indicator obtained from semi-quantitative analysis on SPECT-MIBI images.

We observed that there was a greater likelihood that GBM cases would present abnormal SPECT-MIBI results in relation to tumors of lower grade (Table VI). Histologically, GBMs are characterized by intense vascular proliferation (2), but no association with such data could be confirmed by current immunohistochemical methods due to the limitations cited above.

The results reported in this study must be interpreted with caution due to a number of methodological shortcomings that could limit the internal validity of our study, such as GBM extensive necrosis, MVD quantification and the small number of specimens analyzed. Therefore, it was not possible to fully ascertain whether the use of the SPECT-MIBI approach may serve as an adjunctive resource to the clinical armamentarium.

In conclusion, the results from the present study indicate that the use of MVD, determined immunohistochemically, had a significant relationship with histological grade in the LGAs and AAs, but not in relation to the GBMs. On the other hand, the association between SPECT-MIBI and the MVD of the LGA and AA cases was not significant, nor was the association between SPECT-MIBI and the MVD of the GBM cases.

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