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Original article

Vascularization, Proliferative Activity and the p53 Status in Glioblastomas

Desanka Tasić[1,2], Irena Dimov[3], Miloš Kostov[4], Dragan Dimov[1,2]

[1]*University of Niš, Faculty of Medicine, Niš, Serbia, Retired professor*
[2]*University Clinical Center Niš, Center for Pathology and Pathological Anatomy, Niš, Serbia, Retired pathologist*
[3]*University of Niš, Faculty of Medicine, Department of Immunology, Niš, Serbia*
[4]*Institute of Forensic Medicine, Niš, Serbia*

SUMMARY

Introduction/Aim. Glioblastomas (GBMs) are among the most vascularized human tumors and the presence of microvascular proliferation is one of the diagnostic hallmarks of these malignancies. The aim of the present study was to investigate the extent of vascularization and its relation to proliferative activity and the p53 status in GBMs.
Methods. Tissue samples from 100 selected primary GBMs were analyzed by immunohistochemistry for the expression of CD34 in vascular endothelial cells and Ki-67 antigen (using the MIB-1 antibody) and p53 in tumor cells. The microvessel density (MVD), a measure of the extent of tumor vascularization, was evaluated in CD34-immunostained sections in three hot spots and presented as the mean for each tumor specimen.
Results. We found that the high MVD was more frequent in tumors showing the high MIB-1-labeling index (MIB-1 LI) as compared to those with the low MIB-1 LI, but the difference was not statistically significant. Also, the extent of vascularization did not differ significantly between p53-negative and p53-positive tumors. Both the level of MVD and the proportion of GBMs with low versus high MVD did not differ significantly in relation to the expression levels of p53 (low vs. high or overexpression). No association was found between MVD and tumor cell MIB-1 LI and the p53 status in primary GBMs.
Conclusion. These data suggest that the effect of p53 on primary GBM vascularization failed to detect possibly due to the influence of certain factors, including the presence of other or additional molecular alterations in the tumor cells and the hypoxic microenvironment of tumors. They also support the hypothesis that the effect of p53 on angiogenesis may be tumor-type specific.

Keywords: **glioblastoma, microvessel density, angiogenesis, immunohistochemistry, MIB-1 proliferation index, p53**

Corresponding author:
Desanka Tasić
e-mail: desatasicdim@gmail.com

INTRODUCTION

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults and has a poor prognosis (1, 2). Despite advances in therapy, most patients with GBM die within two years (2, 3), and the overall five-year survival rate is only 5.5% (1). Glioblastomas (GBMs) are highly invasive, hypoxic and hypervascular in nature, and are among the most vascularized human tumors (4). The presence of microvascular proliferation (MVP) is one of the diagnostic hallmarks of GBM (5). Tumors can use several mechanisms to acquire blood supply, including co-option of the pre-existing vasculature by tumor cells, angiogenesis, vasculogenesis, intussusception and vasculogenic mimicry (6). The formation of new blood vessels via the process of angiogenesis (the sprouting of vessels from the pre-existing ones) in GBMs is thought to be crucial for their growth (7). In addition, neovascularization can be supported by other mechanisms such as vasculogenesis (the recruitment of bone marrow-derived circulating endothelial progenitor cells, which differentiate and incorporate into the tumor vessels) (8, 9), and vasculogenic mimicry, in which GBM stem cells transdifferentiate into vascular endothelial cells (10, 11) and vascular mural cells (12).

Neovascularization of brain tumors (particularly in GBMs) is thought to be driven mainly by vascular endothelial growth factor (VEGF) signaling via its vascular endothelial growth factor receptor 2 (VEGFR2) (13). VEGF, a major pro-angiogenic factor, is expressed at high levels in these tumors (4). In GBMs, both tumor hypoxia and genetic alterations (such as EGFR amplification and inactivation of p53, PTEN) together induce the expression of VEGF and other pro-angiogenic factors via hypoxia-inducible factor-1 (HIF-1), resulting in the angiogenic response (7). The HIF-1-independent mechanisms are also implicated in the tumor vessel formation (4, 7). However, the newly-formed vessels are structurally and functionally abnormal (6, 13). Consequently, tumors develop multiple regions of hypoxia and the ensuing foci of palisading necrosis that are linked with adjacent florid MVP (14).

The p53 tumor suppressor protein plays the crucial role in protecting against neoplastic transformation. The p53 protein functions, at least in part, as a transcription factor regulating the expression of target genes that have an important role in mediating cell-cycle arrest, DNA repair, apoptosis, and senescence (15). In addition, the p53 protein has also been attributed to an angiogenesis-regulating function via interference with HIF-1α (the subunit of the heterodimer HIF-1 and a central responder to hypoxia), downregulation of pro-angiogenic factors, including VEGF and basic fibroblast growth factor (bFGF), and upregulation of angiogenesis inhibitors, including thrombospondin-1 (TSP-1), brain-specific angiogenesis inhibitor-1 (BAI-1) and collagen propyl-4-hydroxylase α2 (P4HA2) (16).

Ohgaki et al. reported that mutations of the TP53 gene occurred in two-thirds of precursor low-grade diffuse astrocytomas, having a crucial role in the development of secondary GBMs derived thereof, whereas in primary (de novo) GBMs, TP53 mutations are less frequent (< 30% of cases) (17). The incidence of p53 protein accumulation (nuclear immunoreactivity for p53) is also lower in primary than in secondary GBMs (18).

The aim of the present study was to investigate the extent of vascularization and its relation to proliferative activity and the p53 status in glioblastomas.

MATERIAL

Patients and tissue samples

A series of one hundred selected adult patients with primary GBM (WHO grade IV) which had been diagnosed at the Center for Pathology, Niš, Serbia, between 2004 and 2011, was included in this retrospective study. Patient selection was based on the availability of paraffin-embedded tissue and corresponding clinicopathologic data. None of the GBM patients had undergone radiotherapy or chemotherapy before surgical intervention. All tumor tissue samples were obtained by resection. Tumor specimens from each case were reviewed to confirm the diagnosis of GBM, before inclusion in the study, according to the WHO criteria (5).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were stained with standard hematoxylin and eosin (H&E) for morphologic analysis (the presence of tumor tissue and histopathological criteria of malignancy, including MVP). Representative pa-

raffin blocks for immunohistochemistry (IHC) were selected based on H&E-stained sections. IHC was done on 5-μm-thick deparaffinized sections using the following primary monoclonal antibodies: anti-CD34 (clone QBEnd 10, 1:50, Dako), anti-Ki-67 (clone MIB-1, 1:50, Dako), and anti-p53 (clone DO-7, 1:60, Dako). After the microwave pretreatment, the sections were incubated overnight at 4 °C with the diluted primary antibodies. Detection of immunestaining was performed using standard labeled streptavidin-biotin peroxidase technique (LSAB2 Kit/HRP, Dako) according to the manufacturer's instructions, and diaminobenzidine was used as chromogen. The sections were then counterstained with hematoxylin and mounted.

Evaluation of staining results

The staining results of IHC were evaluated by two investigators (I.D. and D.T.) independently. When the evaluation was different, the final decision was made by consensus. The number of immune-positive endothelial and tumor cells was counted using light microscope (Leica, Germany). Cytoplasmic staining for CD34 and nuclear staining for Ki-67 and p53 were interpreted as being immune-positive.

The MIB-1 labeling index (MIB-1 LI) was obtained by manually counting the positively stained tumor cell nuclei in the areas of their highest density (immunoreactivity for Ki-67 antigen). A total of 1000 tumor cell nuclei were evaluated in each specimen and the percentage of labeled nuclei relative to the total number of tumor cell nuclei was calculated. The mean MIB-1 LI for all GBMs investigated served as the cut-off value.

The p53-positive tumor cell nuclei were also determined by counting 1000 tumor cells in the most stained areas for each specimen. GBMs with more than 10% of p53 stained tumor cell nuclei were estimated as p53-positive, otherwise as negative (19). Immunostaining for p53 in more than 50% of tumor cells (19, 20) was considered as high expression (or overexpression) of the p53 protein, otherwise as low.

Measuring microvessel density

The extent of GBM vascularity was determined quantitatively by measuring microvessel density (MVD). The intratumoral MVD was measured in the CD34 immunostained GBM sections, as described elsewhere (21). The areas of highest vascularization ("hot spots") were selected by scanning the tumor sections at low magnification (x 40 and x 100). MVD was than determined by counting manually all immunolabeled vessels on a x 200 magnification field. In addition to recognizable microvessels, any brown staining of endothelial cell or endothelial cell cluster clearly separated from adjacent microvessels, tumor cells or other connective tissue elements was regarded as a single countable microvessel. Due to the uneven distribution of vascular structures in GBM tissues, the microvessels were counted in three selected fields (hot spots) in each specimen. MVD was expressed as the mean of three hot spots (the mean number of microvessels per x 200 field for each case). The mean MVD for all tumor specimens served as the cut-off: the level of MVD was classified as low if less than the mean, otherwise as high.

Statistical analysis

The statistical analysis was performed using SPSS 16.0 software. All data except for p53 are presented as mean ± standard deviation. The association between MVD and MIB-1 LI and the p53 protein status was determined using the Chi-squared test or the Student's t-test. P values of < 0.05 were considered to be statistically significant.

RESULTS

A series of 100 selected adult patients with primary GBM who underwent tumor resection, without prior adjuvant therapy, were included in the present study. There were 64 males and 36 females. The age of patients ranged from 33 to 78 years with a mean age of 61 years. All tumors analyzed showed microvascular proliferation (endothelial cell proliferation of the various degree was detected on H&E stained sections and by immunostaining of serial sections for Ki-67 antigen and CD34 antigen) (Figure 1). MVP with the formation of glomeruloid vascular structures was observed in most GBM cases (approximately 70%), which were unevenly distributed in tumor tissues.

Microvessel density was a preferred parameter to quantify the extent of vascularization in tumors. In the present study, MVD was evaluated in the CD34-immunostained tumor sections. CD34-positive staining was observed in vascular endo-

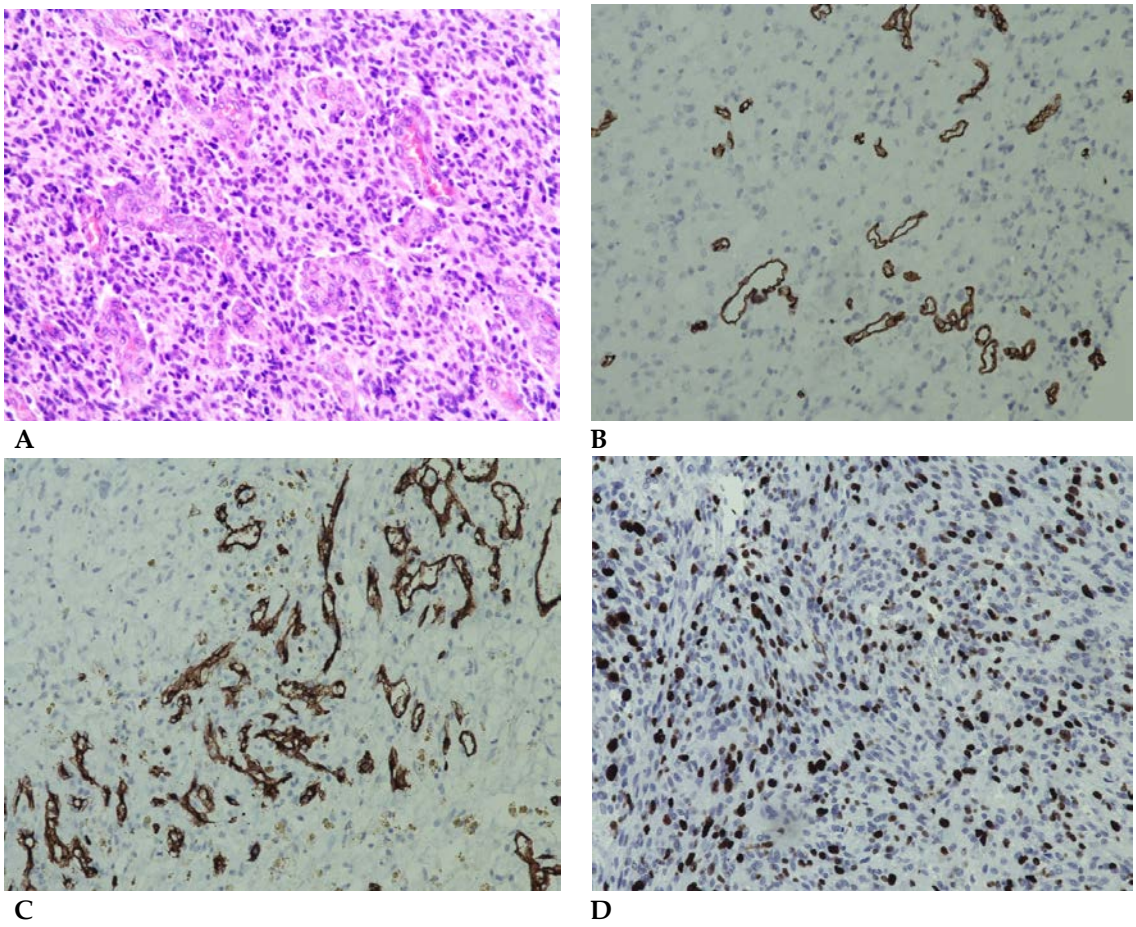


Figure 1. *The presence of microvascular proliferation illustrated by H&E (A) and CD34 immunostained sections (B) of primary glioblastomas. Different microvascular formations illustrated by immunostaining for CD34 (C). Primary glioblastoma with a high MIB-1 labeling index (D). Original magnification x 200 (A-D).*

Table 1. *Association of MVD with MIB-1 labeling index and the p53 status in primary glioblastomas (n = 100)*

Variables	Low MVD		High MVD		P value*
	< 101 (n = 52)		≥ 101 (n = 48)		
MIB-1 LI					
< 27	30	57.7	22	42.3	0.324
≥ 27	22	45.8	26	54.2	
p53 protein status					
p53- negative	22	44.9	27	55.1	0.233
p53- positive	30	58.8	21	41.2	

MVD: microvessel density
Data presented as n (%)
*Chi-squared test

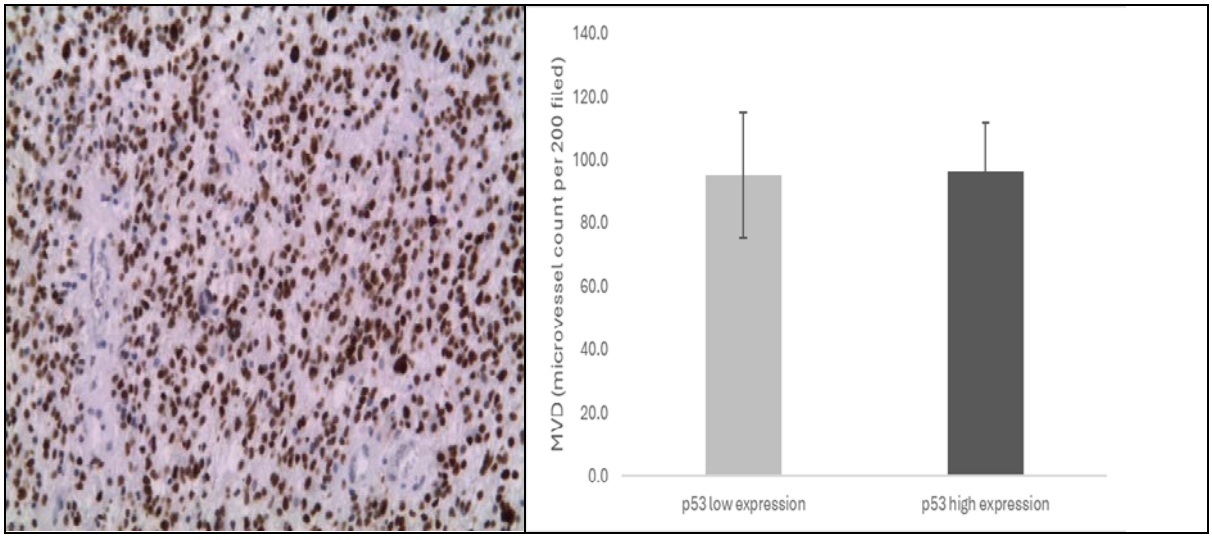
thelial cells, including microvessels, single endothelial cells and endothelial cell clusters (Figure 1B and C). The mean MVD for all primary GBMs was 101.55 ± 23.32 per x 200 microscopic field.

MVD and MIB-1 labeling index and p53 status

The proliferating fraction of tumor cells was evaluated using the MIB-1 antibody for Ki-67 antigen that is expressed in all phases of the cell cycle except GO. The MIB-1 labeling index was expressed as a percentage of positively labeled tumor cell nuclei per total nuclei counted. The mean MIB-1 LI was 27.15 ± 9.41 %. Using the cut-off value of MIB-1 LI, 52% of GBMs having a MIB-1 LI lower than 27% (Table 1). In this group, 30 tumors had the low level of MVD, while 22 had the high level of MVD. Additionally, in the group with the high value of MIB-1 LI (≥ 27%), 22 tumors showed the low level of MVD, while 26 had the high level of MVD (Figure 1, Table 1, Figure 1D). Therefore, the high MVD was more frequent in GBMs with the high MIB-1 LI, whereas the low MVD was more frequent in GBMs with the low MIB-1 LI. However, the difference was statistically not significant (p = 0.324). Consistently, intratumoral microvessel density was not associated with tumor cell MIB-1 labeling index.

Based on the findings that various human cancers carrying TP53 mutations or p53 protein accumulation were more vascularized than those with wild-type p53, and the data that p53 inhbits angiogenesis (16), we investigated the expression of p53 in primary GBMs to evaluate its effect on the extent of vascularization in these tumors. The monoclonal antibody for p53 used in the present study detects both wild- type and mutant p53 proteins. Of initially selected GBM samples with more than 10% of p53 immunostained tumor cell nuclei, 51% of tumors were identified as p53-positive. We found that the proportion of GBMs with a high MVD was larger in the p53-negative group as compared to those in the p53-positive group, but this difference was not statistically significant (p = 0.233) (Table 1).

To further evaluate the effect of the p53 protein status on the GBM vascularization, we excluded p53-negative tumors since they showed the EGFR overexpression (our unpublished data), as known that EGFR amplification and protein overexpression to promote tumor angiogenesis (GBM vascularization). Additionally, we selected tumors with more than 50% of immunostained tumor cell nuclei and this was considered as high p53 expression (or overexpression) (Figure 2A), otherwise as low. The high p53 expression was found in 22 % of GBMs investigated (Table 2). The comparison of MVD (< 101 vs. ≥ 101) with the expression levels of p53 (low vs. high) showed no significant difference



A B

Figure 2. *Primary glioblastoma with a high p53 expression (> 50% tumor cells exhibit the nuclear p53 accumulation). Original magnification x200 (A). MVD (microvessel density) values in tumors with low p53 expression and those with high p53 expression (B). Data are presented as mean ± standard deviation*

Table 2. *Association between MVD and the p53 expression in primary glioblastomas*

Variable	Low MVD		High MVD		P value*
	< 101 (n = 30)		≥ 101 (n = 21)		
p53 expression					
p53 - low	17	58.6	12	41.4	1.000
p53 - high	13	59.1	9	40.9	

MVD: microvessel density
The expression of p53 was divided into two groups: low and high expression (> 50% of immunostained tumor cell nuclei)
Data presented as n (%)
*Chi-squared test

(p = 1.000). In addition, results from the comparative microvessel counts in a low p53-expressing and a high p53-expressing primary GBM specimens failed to show a significant difference (p = 0.856, Figure 2B). Thus, no association was found between intratumoral MVD and the p53 status in primary GBMs.

DISCUSSION

GBMs are highly invasive, aggressive and vascularized malignancies. Despite advances in neurosurgery, radiotherapy and chemotherapy, the prognosis for patients with GBM remains poor (2). The temozolomide (TMZ) chemotherapy and the presence of O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation in GBMs confer with better tumor response to treatment and the survival advantage (22). Both the tumor suppressor protein p53 and MGMT are involved in DNA repair after the chemotherapy or radiotherapy that may contribute to drug resistance (23).

Microvascular proliferation is the defining histopathological phenotype of both primary and secondary GBMs (5, 24). In a series of primary GBMs investigated, the mean intratumoral MVD was found to be high, which reflects the extent of tumor vascularization. The results of our study are in agreement with previously reported data (25-27). Kiesel et al. found that the mean MVD was high in GBM samples with strong fluorescence induced by 5-ALA (5-aminolevulinic acid), which corresponded to compact tumors (27).

Several studies have investigated the proliferative activity in GBMs, especially MIB-1 (Ki-67) labeling index, but mean values of MIB-1 LI varied broadly (28-31). In the present study, the mean MIB-1 LI for primary GBM cases was among the highest as reported in the literature (20, 27-29). In addition, the cut-off value of MIB-1 LI in our study is close to the value reported by others (29). We also analyzed the relation of MVD to MIB-1 LI. The proportion of GBMs with the high MVD was not significantly different between the group with the high MIB-1 LI and the group of tumors with the low MIB-1 LI. There was also no significant difference between the low MVD and MIB-1 LI values (low vs. high). These data demonstrate that there is no association between MVD and tumor cell MIB-1 labeling index in a series of primary GBMs investigated. This finding is in agreement with data in previous studies (20, 25, 32). Since the tumor angiogenesis is a highly complex process that has both a genetic and hypoxic regulation (involving both the tumor cells and the associated microenvironment), it is possible that the extent of tumor vascularization does not correlate with the tumor cell proliferation as noted in the present study and studies mentioned above.

In addition to the crucial tumor suppressive function, the p53 protein has been attributed a role in the regulation of tumor angiogenesis (16). Mutation of the TP53 gene is common in GBMs, seen in 60-70% of secondary GBMs and 25-30% of primary GBMs (33). These TP53 mutations are associated with a poor prognosis for overall survival in GBM patients (23). Most TP53 mutations in GBMs are missense mutations (34), which lead to stabilization and nuclear accumulation of mutant p53 proteins, but the incidence of p53 protein accumulation is more frequent than TP53 mutations are (18). As previously reported (23), this discrepancy may be explained by the complex formation of p53 protein with other proteins (oncoproteins) that stabilize or

modify it. Therefore, the cut-off value (> 50%) for p53 was used in the present study, considering that the tumors with possible TP53 mutations show a high positive ratio (> 50%) for p53 (35). TP53 mutations are most common in the DNA-binding domain, and generally result in loss-of-function, gain-off-function and dominant -negative (mutational) effect for p53 (33). Several studies have demonstrated that mutant p53 possesses gained (oncogenic) functions, contributing to tumor growth and progression (23, 36). Also, TP53 mutation may decrease the chemosensitivity of GBM to TMZ by increasing MGMT expression (23).

To evaluate the effect of the p53 status on vascularity in GBMs, we analyzed the relation between MVD and p53 expression (p53-negative vs. p53-positive tumors, and ones with low vs. high p53 expression). The proportion of GBMs with a high MVD was larger in the p53-negative group as compared to the p53-positive, but this difference was not statistically significant. We found that the level of MVD in tumors with a high p53 expression (> 50%), which was suggestive for the presence of TP53 mutations, was not significantly higher than that in tumors with a low p53 expression. Additionally, the proportion of GBMs with a high MVD was not statistically different between low p53- and high p53-expressing tumors.

The present study revealed that the extent of vascularization in primary GBMs was not associated with the p53 protein status. These data are in line with previous findings from other studies (20, 37). Berger et al. investigated the value of the TP53 mutational status on the extent of vascularization in primary GBMs, and were found that neither total area nor total number of vascular structures differed significantly between p53 wild-type and p53 mutant tumors (37). Additionally, among the investigated angiogenesis-related target genes (VEGF, bFGF,TSP-1, BAI1, P4HA2), only P4HA2 mRNA was found to be upregulated by wild-type p53 overexpressed in LN-308 GBM cells but without increasing in protein levels, indicating that the p53/P4HA2-mediated anti-angiogenic pathway is defective in GBM cells, which is opposite to H1299 cancer cells (37). These data suggest that the effect of p53 on tumor angiogenesis may be cell or tumor-type specific and challenge the view of p53 as an angiogenesis-regulating factor in GBM (37).

Notably, in a study of diffuse low-grade astrocytomas, it was observed that MVD and absolute vessel number were increased in TP53 mutated tumors in comparison to their TP53 wild-type counterparts, indicating that p53 exerts an angiogenesis-inhibiting function in diffuse low-grade astrocytomas (38). Given that the TP53 mutations stand for an early event in the progression of astrocytomas (17, 18) and the formation of new blood vessels (neovascularization) characterizes the phenotype of GBMs (5), it seems that the influence of p53 on tumor angiogenesis may decrease during malignant progression with accumulated of additional molecular alterations in tumor cells and tumor hypoxia.

GBMs are known to be highly vascularized tumors with potent angiogenic activity (4,7). One common feature in the transition from low-grade or anaplastic astrocytomas to secondary GBM is a dramatic increase in MVP (24). An equivalently robust MVP is observed in primary GBM (24). Accordingly, several studies have demonstrated that MVD increased with an increase in the atrocytoma grade, being the highest in grade IV tumors (21, 25, 32, 39). The MVD was also observed to increase with an increase in pathologic grade of gliomas (predominantly astrocytomas) investigated (40). However, studies concerning the prognostic value of MVD in malignant astrocytomas (mainly GBMs) disclosed controversial results. Some studies revealed that intratumoral MVD was an independent prognostic factor (21, 39). In contrary, other studies revealed no prognostic value of MVD (41-43).

Besides the high MVD, GBMs usually present with a regionally heterogeneous vascularization. Therefore, in the present study, the MVD was evaluated in three hot spots and presented as the mean for each tumor specimen. The vascular patterns have been recognized as prognostic factor for GBMs (20). A large study of primary GBMs revealed a significant correlation of vascular patterns with patient outcome (20). It was observed that prominent classic (predominantly capillary-like) vascular pattern and low content of bizarre vascular pattern (predominance of glomeruli/garland/vascular clusters) was an independent factor for longer survival (20). It was also observed that in tumors with prominent classic vascular pattern, MVD was significantly higher though the MIB-1 LI did not differ significantly. In a systematic study of two large GBM series, both vascular parameters (MVD and vascular patterns) were evaluated retrospectively by multiple observers (42). MVD and vascular patterns were not

correlated with patient outcome. The authors also concluded that poor observer agreement limits the clinical utility of MVD and vascular patterns as prognostic factors and recommended that these vascular parameters need to validated (42).

The data concerning p53 suggest that it is not only involved in regulating tumor angiogenesis (16) but it can also been potentially implicated in tumor response to anti-angiogenic therapy (AAT) (44). Experimental studies in mice bearing tumors derived from TP53-/- colorectal cancer cells were showed to be less responsive to AAT than mice bearing isogenic TP53+/+ tumors (44). Moreover, the study concerning p53 showed that p53-positive microvascular proliferation cells exhibit TP53 mutations (identical to those in tumor cells) and tend to be clustered histopathologically in GBM tissues (35). These findings are in keeping with previous studies showing that GBM stem cells are capable of transdifferentiation into endothelial cells (10, 11) and mural cells (12) to promote vasculogenic mimicry (VM). It has been shown that the endothelial-like cells carry the same genetic alterations as tumor cells within GBM (such as TP53 mutation or EGFR amplification) (10, 11) and that anti-angiogenic treatments such as VEGF blockade can only partially inhibit this vasculogenic mimicry (11). Interestingly, one study showed that in high-grade astrocytomas, the level of MVD was lower in VM-positive tumors than those in VM-negative tumors (43). Findings from previous studies suggest that VM as an alternative mechanism in GBM vascularization may reduce responsiveness to AAT. Anti-angiogenic therapies were used (as monotherapy or combined with chemoradiotherapy) mainly against VEGF and its receptors to normalize the tumor vasculature in patients with GBM, however, with no significant survival benefit to patients (13, 45).

CONCLUSION

In the present study of 100 selected primary GBMs, the mean intratumoral MVD evaluated in three hot spots was found to be high, which reflects the extent of vascularization in these malignancies. No association was found between MVD and tumor cell MIB-1 labeling index and the p53 status. The extent of vascularization did not differ significantly between p53-negative and p53-positive tumors or between ones expressing low and high levels of p53. These findings suggest that the effect of p53 on primary GBM vascularization failed to detect possibly because of the influence of certain factors, including the presence of other or additional molecular alterations in the tumor cells and the hypoxic microenvironment of tumors. However, our study has some limitations since it was retrospective and obtained data concerning the p53 status based on immunohistochemistry. In this context, further studies will be required.

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Vaskularizacija, proliferativna aktivnost i p53 status u glioblastomima

Desanka Tasić[1,2], Irena Dimov[3], Miloš Kostov[4], Dragan Dimov[1,2]

[1]*Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija, profesor u penziji*
[2]*Univerzitetski klinički centar Niš, Centar za patologiju i patološku anatomiju, Niš, Srbija, patolog u penziji*
[3]*Univerzitet u Nišu, Medicinski fakultet, Departman za imunologiju, Niš, Srbija*
[4]*Institut za sudsku medicinu, Niš, Srbija*

SAŽETAK

Uvod/Cilj. Glioblastomi (GBM) spadaju u najviše vaskularizovane tumore kod ljudi, a prisustvo mikrovaskularne proliferacije predstavlja jedno od dijagnostičkih obeležja ovih maligniteta. Cilj ove studije bio je da ispita stepen vaskularizacije u odnosu na proliferativnu aktivnost i p53 status GBM-a.
Metode. Imunohistohemijski je u uzorcima tkiva 100 odabranih primarnih GBM-a analizirana ekspresija CD34 u vaskularnim endotelnim ćelijama, Ki-67 antigena (primenom MIB-1 antitela) i p53 u tumorskim ćelijama. Mikrovaskularna gustina (MVG), mera stepena vaskularizacije tumora, određivana je na CD34-imunobojenim presecima u trima „vrućim tačkama" i prikazana kao prosečna vrednost za svaki uzorak tumora.
Rezultati. Visok MVG je bio češći nalaz u tumorima sa visokim MIB-1 indeksom nego u onima koji su imali nizak MIB-1 indeks, ali razlika nije bila statistički značajna. Ni razlika u stepenu vaskularizacije između p53-negativnih i p53-pozitivnih tumora nije bila značajna. Nivo MVG-a i proporcija GBM-a sa niskim odnosno visokim MVG nisu se značajno razlikovali u odnosu na nivo ekspresije p53 (nizak odnosno visok). Nije zapažena povezanost MVG sa MIB-1 indeksom tumorskih ćelija i p53 statusom u primarnim GBM-ima.
Zaključak. Dobijeni podaci ukazuju na to da efekat p53 na vaskularizaciju primarnih GBM-a nije bio detektovan; možda su na to uticali određeni faktori, uključujući prisustvo drugih ili dodatnih molekularnih alteracija u tumorskim ćelijama i hipoksične mikrosredine tumora. Takođe, ovi rezultati podržavaju hipotezu o tome da pomenuti efekat p53 može biti specifičan za tip tumora.

Ključne reči: **glioblastom, mikrovaskularna gustina, angiogeneza, imunohistohemija, MIB-1 proliferativni indeks, p53**