ERG is a novel and reliable marker for endothelial cells in central nervous system tumors

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Abstract. ETS-related gene (ERG) is a transcription factor that has been linked to angiogenesis. Very little research has been done to assess ERG expression in central nervous system (CNS) tumors. We evaluated 57 CNS tumors, including glioblastomas (GBMs) and hemangioblastomas (HBs), as well as two arteriovenous malformations and four samples of normal brain tissue with immunohistochemistry using a specific ERG rabbit monoclonal antibody. In addition, immunostains for CD31, CD34, and α-smooth muscle actin (α-SMA) were performed on all samples. CD31 demonstrated variable and sometimes weak immunoreactivity for endothelial cells. Furthermore, in 1 case of a GBM, CD34 stained not only endothelial cells, but also tumor cells. In contrast, we observed that ERG was only expressed in the nuclei of endothelial cells, for example, in the hyperplastic vascular complexes that comprise the glomeruloid microvascular proliferation seen in GBMs. Conversely, α-SMA immunoreactivity was identified in the abluminal cells of these hyperplastic vessels. Quantitative evaluation with automated methodology and custom Matlab 2008b software was used to calculate percent staining of ERG in each case. We observed significantly higher quantitative expression of ERG in HBs than in other CNS tumors. Our results show that ERG is a novel, reliable, and specific marker for endothelial cells within CNS tumors that can be used to better study the process of neovascularization.

Introduction

Angiogenesis plays a critical role in various pathologic processes, such as in the pathogenesis of ischemic and neoplastic disorders, including central nervous system (CNS) tumors [1]. For example, in CNS tumors, angiogenesis plays a crucial role in both growth and progression [2]. In addition, the presence or absence of florid microvascular proliferation is an important criterion used in the grading of fibrillary astrocytomas [3] and anti-angiogenesis is one of the therapeutic approaches used in high-grade gliomas [4]. Various CNS tumors, including hemangioblastomas (HBs) and glioblastomas (GBMs), are highly vascularized [1]. In many tumors, hypoxia inducible factor-1α (HIF-1α) is regulated by oxygen concentration and is involved in the activation of many genes, including genes that play a role in survival in anaerobic conditions, as well as angiogenesis [5]. In both HBs and GBMs, the accumulation of HIF-1α leads to increased angiogenesis primarily through the upregulation of vascular endothelial growth factor (VEGF) [1, 6]. For example, in GBMs, the accumulation of HIF-1α protein causes the upregulation of VEGF mRNA in hypoxic pseudopalisading cells adjacent to areas of necrosis [1]. In HBs however, the decreased degradation and subsequent accumulation of HIF-1α protein is caused by a loss of function of the von-Hippel Lindau (VHL) tumor suppressor protein [7], which causes the upregulation of VEGF mRNA in stromal cells [1, 6].

ETS-related gene (ERG) is a transcription factor whose expression in normal physiologic conditions is found in endothelial cells and cells of hematopoietic lineage [8]. ERG plays a role in endothelial cell migration and has been linked to angiogenesis [9]. For example, a recent study demonstrated that RhoJ, a Rho GTPase family member highly restricted to endothelial cells in several tissues, is a downstream target of ERG and plays a role in capillary morphogenesis, an important step of the angiogen-
ic cascade [10]. ERG also interacts with other transcription factors in order to regulate various genes that are expressed within the endothelial cell lineage, including VE-cadherin, angiopoietin-2, and von Willebrand Factor (vWF) [8]. Moreover, ERG inhibition leads to endothelial cell apoptosis, as well as a decrease in the total number of endothelial cells, endothelial cell-cell connections, and vascularization [11].

Much has previously been done to assess ERG expression in endothelial cells within vascular lesions. For instance, one recent study demonstrated strong endothelial immunoreactivity for ERG in both benign and malignant vascular tumors, as well as other vascular lesions, including arteriovenous malformations (AVMs) and papillary endothelial hyperplasia [12]. Furthermore, ERG has previously been shown to be both a sensitive and specific marker for endothelial cells in various vascular malignancies, including angiosarcoma, hemangioma, lymphangioma, Kaposi sarcoma, and hemangioendothelioma [13]. Evidence has also demonstrated the presence of ERG overexpression within various non-vascular neoplasms, including prostate carcinoma, Ewing’s sarcoma, and acute myeloid leukemia [14, 15, 16, 17]. However, a review of the literature indicates that very little has been done to assess the expression of ERG in CNS tumors, or to compare its reliability with that of other endothelial markers, such as CD31 and CD34. Using immunohistochemistry, and a specific rabbit monoclonal antibody, we evaluated ERG expression in CNS tumors. In addition, immunostains for CD31, CD34, and α-smooth muscle actin (α-SMA) were performed on all samples. We also implemented a quantitative analysis of ERG expression throughout different tumor types using a novel computational methodology via a custom Matlab 2008b program. Overall, our results suggest that ERG is a novel, reliable, and specific marker for endothelial cells in CNS tumors that can be used to better study the process of neovascularization.

**Materials and methods**

**Tissue samples**

This Health Insurance Portability and Accountability Act-compliant study was
conducted under a protocol approved by the Institutional Review Board of New York University School of Medicine. We evaluated 57 CNS tumors, which included 16 GBMs, of which 1 case was a recurrent high-grade glioma post-radiation therapy; 4 anaplastic astrocytomas (AAs), 8 HBs, 12 meningiomas, 8 metastatic carcinomas, 2 oligodendrogliomas (OGs), 2 hemangiopericytomas (HPCs), 2 solitary fibrous tumors (SFTs), and 3 schwannomas classified according to the World Health Organization (Table 1); as well as 2 AVMs. The tumors were from 30 female and 27 male patients, with an age range of 19 – 84 years (mean age 53.4). 39 tumors were supratentorial and 18 were infratentorial. Four samples of normal brain tissue removed in the course of surgical exposure were used as controls. When present, normal brain tissue adjacent to the tumor was also used as an internal control.

**Table 1.**

| Age | Sex | Location                        | Pathology          |
|-----|-----|---------------------------------|--------------------|
| 56  | F   | Right cerebello-pontine angle   | Schwannoma         |
| 71  | F   | Left suboccipital region        | Solitary fibrous tumor |
| 84  | F   | Right suboccipital region       | Solitary fibrous tumor |
| 20  | M   | Right frontal lobe              | Normal brain tissue |
| 63  | M   | Right frontal lobe              | Normal brain tissue |
| 63  | M   | Right frontal lobe              | Normal brain tissue |
| 68  | F   | Left parietal lobe              | Normal brain tissue |

**Immunohistochemistry**

Serial sections were stained for hematoxylin and eosin (H & E) and immunostained with a rabbit monoclonal antibody for ERG (clone EPR3864; 0.8 mg/mL). In addition, mouse monoclonal antibodies were also used to stain sections for CD34 (clone QBEnd/10; 23 mg/mL), CD31 (clone JC70; 0.65 mg/mL), and α-SMA (clone IA4; 0.02 mg/mL). Heat-induced epitope retrieval was done by boiling the deparaffinized tissue sections in 10 mmol/L citrate buffer (pH 6.0) in a 1,200 W microwave oven at 90% output for 64 minutes for ERG, 36 minutes for both CD34 and CD31, and 8 minutes for α-SMA. The sections were allowed to cool to room temperature for 30 minutes and subsequently incubated with secondary antibodies at room temperature overnight on a NexES automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA). We used an anti-rabbit biotinylated goat secondary antibody for ERG and one that was anti-mouse for CD34, CD31, and α-SMA. All primary and secondary monoclonal antibodies were purchased prediluted from Ventana Medical Systems. For each antibody, horseradish peroxidase-conjugated strepavidin with 3,3′-diaminobenzidine was used as the chromogen. Nuclei were lightly counterstained with hematoxylin, and slides were dehydrated and mounted with permanent medium. For each immunostain, control procedures included isotype-matched rabbit and mouse monoclonal antibodies.

**Matlab quantitative analysis of ERG expression**

In each sample of tumor and normal brain tissue, the section immunostained for ERG was evaluated using light microscopy at 100× magnification. The two foci containing the most ERG stained capillaries and microvessels, or “hot spots” within each section were located and used for analysis [18]. Computational analysis of ERG expression was performed using a routine spectral clustering threshold method with custom Matlab 2008b software [18], which provided a pixel count quantification of the presence of the immunostain in each section. Pix-

![Figure 1. Matlab quantitative determination of EVI. a: Original image of a GBM stained with ERG (100× magnification). b: Demonstration of calculation of degree of ERG staining (EVI = 5.028).](image)
for quantitative analysis and comparison of different pathologies. The mean EVI for each tumor type and normal brain tissue was calculated and plotted. Statistical analysis with the nonparametric Mann-Whitney test was used to compare percent staining across tumor types and normal brain tissue.

**Results**

**Immunohistochemical evaluation of gliomas**

In all 15 GBMs, all 4 AAs, and the 2 OGs, we observed strong nuclear immunoreactivity for ERG exclusively in endothelial cells lining vascular lumens (Figure 2b, 3b, 4b). For example, in the glomeruloid microvascular proliferation composed of hyperplastic vascular complexes adjacent to pseudopalisading cells surrounding areas of necrosis, ERG was only detected in endothelial cells (Figure 3b). In contrast, α-SMA immunoreactivity was detected within the abluminal cells of hyperplastic vessels in GBMs (Figure 2e, 3e, 4d). In the 1 GBM case where microvascular proliferation was absent, endothelial cells were also highlighted by the ERG immunoreactivity. In the post-irradiated GBM, secondary microvascular changes were present and with endothelial cells that were strongly reactive for the ERG immunostain. In GBMs, AAs, and OGs immunoreactivity for CD31 and α-SMA was variable and sometimes weak or even absent within non-hyperplastic vascular channels (Figure 2d, e, 3d, e, 4d), while immunoreactivity for CD34 was moderate (Figure 2c, 3c, 4c). In partially sclerosed vessels α-SMA immunoreactivity was reduced, whereas ERG immunoreactivity was present. In addition, in 1 GBM where ERG only stained endothelial cells (Figure 4b), CD34 stained both endothelial and tumor cells (Figure 4c). ERG-positive endothelial cells were seen at the invasive edge of all GBMs as well.

**Immunohistochemical evaluation of HBs**

The 8 HBs were highly vascular (Figure 5a). In every case, large areas of tumor showed an anastomosing network of vessels that sepa-
rated variably abundant groups of stromal cells (Figure 5a). In all 8 HBs, like in GBMs, ERG was only expressed in endothelial cells lining vascular lumens, demonstrating markedly diffuse neovascularization, but was not expressed in stromal cells (Figure 5b). Unlike ERG, CD31 showed variable and sometimes weak immunoreactivity within endothelial cells (Figure 5d), while CD34 showed moderate immunoreactivity (Figure 5c). In contrast to ERG, like in GBMs the α-SMA immunostain highlighted abluminal smooth muscle cells within vessels (Figure 5e).

**Immunohistochemical evaluation of AVMs, HPCs, meningiomas, metastatic carcinomas, schwannomas, and SFTs**

Like in gliomas and HBs, in AVMs, HPCs, meningiomas, metastatic carcinomas (Figure 6a), schwannomas, and SFTs, the nuclei of the endothelial cells lining vascular lumens demonstrated strong immunoreactivity for ERG (Figure 6b). Here again, like in GBMs, AAs, and HBs, endothelial cells were only variably immunoreactive for CD31, and immunoreactivity for CD34 was more intense than for CD31 (Figure 6c). We observed variable α-SMA immunoreactivity within the walls of the vascular channels.

In the 4 control normal brains, and in cerebral and cerebellar tissue adjacent to 12 GBMs, 3 AAs, and 4 HBs, detectable ERG, CD31, and CD34 immunoreactivity was seen in endothelial cells lining vascular lumens. Here again there was stronger immunoreactivity for ERG as compared to CD31, CD34, and α-SMA. α-SMA immunoreactivity was also observed in the media of arteries and arterioles in the 4 control normal brains, as well as in normal brain distant from 1 GBM and 1 AA. For each tumor case and sample of normal brain tissue used in this study, no staining was observed with isotype-matched rabbit and mouse monoclonal antibody controls in the absence of primary antibody.

**Matlab quantitative analysis of ERG expression**

The results of the quantitative analysis of ERG immunoreactivity are summarized in Figure 7 and Table 2. We demonstrated significantly more extensive ERG expression in HBs than in other CNS tumors, including GBMs (threshold for statistical significance p < 0.05). Meningiomas and GBMs had the fourth and fifth greatest mean EVIs respectively. As expected, mean EVI was lowest in normal brain tissue. Schwannomas were demonstrated to have the lowest mean EVI of the tumors sampled within our study, and were not found to have significantly more extensive immunostaining for ERG than normal brain tissue. In contrast, meningiomas, metastatic carcinomas, and AAs were found...
to have significantly more extensive immunostaining for ERG than normal brain tissue.

**Discussion**

**ERG is a novel, reliable, and specific marker for endothelial cells within CNS tumors**

Our studies demonstrated that in contrast to ERG, CD31 only variably highlighted endothelial cells within CNS tumors and sometimes demonstrated a notably weaker endothelial immunoreactivity. CD31, or platelet endothelial cell adhesion molecule, is a transmembrane glycoprotein expressed in normal physiologic conditions by endothelial cells, platelets, and blood leukocytes, and whose functions include cellular adhesion, platelet activation, and angiogenesis [18, 20]. CD31 is one of the most frequently utilized immunohistochemical markers for endothelial cells, for example, as a marker of angiogenesis in the settings of atherosclerosis and abdominal aortic aneurysm [21], for the quantitative analysis of blood vessels [22], and for determining the degree of neovascularization in a variety of neoplasms, including cervical cancer, ovarian cancer, and Kaposi sarcoma [22, 23, 24].

However, in spite of the ubiquitous use of CD31 as a marker for endothelial cells, this immunostain suffers from various shortcomings. For instance, CD31-positive immunostaining has been reported as a less sensitive marker of microvascular density than other markers within neoplasms such as cervical cancer [22]. Furthermore, the expression of CD31 in platelets and blood leukocytes that are adherent to vascular walls may lead to their misidentification as endothelial cells, thus reducing the specificity of this particular immunostain. Additionally, in our study we observed that CD31 only variably and weakly highlighted endothelial cells within CNS tumors (Figure 2d, 3d, 5d), calling into question this immunostain’s use as a marker of such cells.

CD34 is yet another immunostain with widespread utilization as a marker for endothelial cells. CD34 is a transmembrane glycoprotein expressed in normal physiologic conditions by endothelial cells and hematopoietic stem cells, as well as in dural fibroblastic lesions and non-neoplastic fibrous/leptomeningeal lesions, and whose functions include control of differentiation of stem cells and adhesion [25]. Like CD31, CD34 has been proposed as a sensitive marker for endothelial cells [22], has been used to diagnose vascular tumors [26], and has been used to evaluate the degree of angiogenesis in a variety of neoplasms, including cervical cancer, prostate cancer, and multiple myeloma [22, 27, 28].

However, like CD31, CD34 is affected by several drawbacks which should allow us to question the prevalence of its use as an endothelial cell marker. For instance, CD34-positive immunostaining has also been reported in non-vascular cells within CNS tumors, including solitary fibrous tumor and ganglioglioma [29, 30, 31], thus limiting the use of CD34 as a specific marker for endothelial cells. In addition, in 1 case of a GBM in our study, CD34 highlighted not only endothelial cells, but also tumor cells (Figure 4c).

In our study we have demonstrated that unlike CD31 and CD34, ERG is exclusively expressed in endothelial cells within CNS tumors, lending support to the notion that ERG is a more specific marker for such cells.
Furthermore, ERG dependably and intensely highlighted endothelial cells in CNS tumors (Figure 2b, 3b, 4b, 5b, 6b), providing solid evidence that ERG is a more robust endothelial marker than CD31 and CD34 are. In line with these observations and given the various limitations of the CD31 and CD34 immunostains, we recommend that ERG should be used in the future as the primary endothelial immunostain for CNS tumors.

**Quantitative expression of ERG in endothelial cells in CNS tumors**

Our results revealed significantly higher ERG expression in HBs than in other CNS tumors, including GBMs, which had the fifth greatest mean EVI. These results are consistent with the diffuse, increased vascular density seen in HBs [1], which contrasts with the multifocal and patchy microvascular proliferation in GBMs, for example, adjacent to areas of necrosis [1]. Therefore, although HBs and GBMs are both highly vascularized, the differences in their mean EVI values may be explained by variations in the overall respective homogenous and heterogeneous distribution and landscape of neovascularization within such tumors. The findings that GBMs had a higher mean EVI than AAs and that both GBMs and AAs had a higher mean EVI than normal brain tissue are consistent with the microvascular proliferation seen within high grade gliomas and compatible with the grade assigned to these neoplasms, for the presence or absence of florid microvascular proliferation is an important criterion used in the grading of gliomas [3]. Similarly, meningiomas and metastatic carcinomas of the brain, in contrast to schwannomas, were found to have a significantly higher mean EVI than normal brain tissue. Our results regarding meningiomas and metastatic carcinomas are in line with the important role that angiogenesis plays in such neoplasms [32, 33], providing further evidence that mean EVI correlates with endothelial cell number within CNS tumors. As benign nerve sheath tumors, schwannomas are less likely to have marked angiogenesis than malignant peripheral nerve sheath tumors, also compatible with our results [34].

**Use of ERG in understanding the process of neovascularization in gliomas**

In our study we observed that ERG was only expressed in the nuclei of endothelial cells lining vascular lumens in normal brain tissue and within CNS tumors, for example, in the glomeruloid microvascular proliferation seen in GBMs. In contrast, α-SMA immunoreactivity was identified in abluminal
cells within the hyperplastic vascular complexes of GBMs [35]. Clearly, the accurate delineation of the cellular components taking part in the microvascular proliferation seen in GBMs is important in order to better understand angiogenesis in CNS tumors. One unresolved and still debated issue related to the cellular components contributing to hyperplastic vessels within GBMs continues to exist. Some have shown that only endothelial cells without the involvement of smooth muscle cells are involved in the microvascular proliferation seen in GBMs [35]. In contrast, other studies have provided experimental data indicating that both endothelial and smooth muscle cells are involved in the microvascular proliferation leading to vascular hyperplasia within glial neoplasms [36]. Our results, which demonstrate the presence of both ERG and α-SMA immunostained cells within vascular lumens, provide novel support for the latter hypothesis of a mixed dual cellular component involved in the glomeruloid microvascular proliferation seen in GBMs, consisting of both endothelial and smooth muscle cells.
Conclusion

In conclusion, we have shown that ERG is a novel and more reliable marker for endothelial cells within CNS tumors than CD31 and CD34 are, adding another tool to the arsenal for the evaluation of CNS tumors. Furthermore, we have demonstrated that ERG expression is significantly higher in HBs than in other types of CNS tumors, including GBMs. Our results help to elucidate the cellular component of the microvascular proliferation of GBMs, furthering our understanding of the development of angiogenesis in CNS tumors. Future studies involving the ERG immunostain may be undertaken in order to better define the biological mechanisms that underlie the process of neovascularization in CNS tumors.

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Conflict of interest

None of the authors reports a conflict of interest.

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