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Increased Endothelial Progenitor Cells with Age and Grade of Malignancy in Astrocytic Glioma Patients

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ABSTRACT

Astrocytic gliomas are the most common primary brain tumours that originated from human glial cells. The tumours rely upon endothelial progenitor cells (EPCs) for neoangiogenesis. This study aimed to investigate the association between tissue resident EPCs in a brain tumour and normal adjacent tissue in relation to age and grade of astrocytic glioma. Astrocytic glioma patients (n=22), grade I to grade IV were consented from Hospital Universiti Sains Malaysia. Brain tumour tissue and normal adjacent brain tissue samples were obtained from each patient during surgery. The EPCs were stained with $CD133^+$ and $VEGFR\text{-}2^+$ markers. The tissue residents EPCs for each sample were determined using the immunofluorescence microscopy method. The age of the patients increased by disease severity in the following order (Grade I: 21.33±20.79 years) < (Grade II: 46.50±0.707 years) < (Grade III: 47.38±11.95 years) < (Grade IV: 48.44 ±10.66 years). The EPCs in brain tumour correlated significantly with the age of the patients with positive correlation (Spearman's rho correlation test, r=0.52; p=0.013). The tissue resident EPCs in the brain tumour (median=0.40, IqR=0.59) were significantly higher compared with the adjacent normal brain (median=0.067, IqR=0.29) (Wilcoxon Signed-Rank Test, Z stat=-3.587, p<0.001). Higher tissue resident EPCs were found in high grade (III & IV) glioma compared with EPCs in low grade (I & II) glioma (median=0.61, IqR=0.70 vs. median=0.26, IqR=0.30; z=-1.763 p=0.078). This study showed increased EPCs with age and

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grade of malignancy in astrocytic glioma patients. Therefore, targeting EPCs in gliomas based on tumour grade malignancy and age of the patients might be useful in effective treatment of astrocytic glioma.

Keywords: Astrocytomas, brain tumours, endothelial progenitor cells, glioma

INTRODUCTION

According to WHO classification of central nervous system tumours, astrocytomas are typically classified as pilocytic (Grade I – less aggressive), diffuse (Grade II), anaplastic (Grade III) or glioblastoma multiforme (Grade IV – most aggressive) in order of increasing anaplasia (Okada et al*.,* 2009). Glioblastoma multiforme is the most common type of astrocytoma and it is a highly invasive and almost uniformly fatal tumour. Glioblastoma multiforme is among the most highly vascularіaed of all malignancies and relies upon angiogenesis for growth and histological progression (Vredenburgh et al*.,* 2009). The Grade IV gliomas are cytologically malignant, mitotically active and necrosis-prone neoplasms and they are related typically with formation of rapid pre- and post-operative disease. A propensity for craniospinal dissemination and infiltration of surrounding tissue characterises Grade IV tumours. Such tumours also have endothelial proliferation with apparent multi-layering of endothelium and glomeruloid microvascular proliferation (Louis et al*.*, 2007).

Grade III tumours (anaplastic astrocytoma) are commonly found with lesions and histological malignancy, including nuclear atypia and brisk mitotic activity. The treatment options for Grade III glioma are either by radiation or chemotherapy or a combination of both. Diffusely infiltrative astrocytic tumours with cytological atypia is a characteristic of Grade II tumours (diffuse astrocytoma). The Grade II *neoplasms* are generally *infiltrative and* always recur despite low-level proliferation. They also have the tendency to develop into higher grades i.e. from diffuse astrocytoma (low grade) that proliferates into anaplastic astrocytoma and glioblastoma. The Grade I gliomas are assigned into more circumscribed pilocytic astrocytoma. They carry properties of low proliferative potential of lesions and chances of curability by surgical resection (Louis et al*.*, 2007).

The endothelial progenitor cell (EPC) acts as an angiogenic precursor. *EPCs* probably have a great *potential* in pathophysiology and treatment of brain cancer (Gao et al*.*, 2010). The number of EPCs that are found in patients with glioma varies by grade of the malignancy and age of the patients. A *previous study reported* that patient with glioma Grade IV and Grade III who have undergone treatment (radiotherapy or chemotherapy) showed significant reduction of circulating EPCs compared with healthy controls (Corsini et al*.*, 2012). In addition, the age of the patients also affects tumour pathophysiology in astrocytic gliomas (Rebetz et al*.*, 2008). Therefore, targeting EPCs in gliomas by considering the grade and age of the patients might be beneficial in effective antiangiogenic treatment and will serve as potential new targets or vectors for adjuvant therapy (Yu et al*.*, 2010).

Significant reduction of EPCs showed impaired and delayed growth in tumours followed by reduction in tumour vessel density. The study showed that the inhibition of EPC might block the neoangiogenesis process particularly in the growing tumours after incomplete surgical removal of primary lesion or treatment (Nolan et al., 2007). During vascular injury, circulating host-derived endothelial cells are highly demanded for neoangiogenesis (Briasoulis et al., 2011; Leone et al., 2009; Yin et al., 2010). Moreover, with advancing age, it is likely that oxidative cellular damage accumulates in EPCs and deteriorates its function (He et al., 2009). Therefore, it is reasonable to *believe that* the number of EPCs and age determine the severity of astrocytic glioma patients. In this study, the associations between tissue resident EPCs and age and grade of glioma were investigated.

METHOD

Patient Recruitment

Astrocytic glioma patients (n=22) were consented from the Hospital of Universiti Sains Malaysia (HUSM). Only cases that confirmed with WHO grading (I to IV) of astrocytic glioma were included in the study. Ethical approval to conduct this study was obtained from the Human Research Ethics Committee, Universiti Sains Malaysia (FWA Reg No: 00007718; IRB Reg. No: 00004494).

Tissue Analysis for EPCs

Microsurgical specimens of brain tumour and adjacent normal brain tissue were obtained from each patient for the analyses of tissue resident EPCs. Tissues were analysed using immunofluorescence staining. The total number of tissue resident EPCs in the tumour and adjacent normal brain tissue specimens were characterised using immunofluorescence microscopy.

In the IHC technique, tissue biopsies were fixed in 10% paraformaldehyde and processed for histology and immunohistochemistry analyses. The antibodies that were used in this study included CD133 (clone ACC133/1) and vascular endothelial growth factor-2 (VEGFR-2) to characterise the tissue resident EPCs (Hilbe et al., 2004; Rafat N., 2010; Toshner M., 2009).

Slide Preparation

Tissue biopsy was fixed in paraformaldehyde 10% and three sections from each brain tissue sample was cut at an interval length of about 6.5 mm with a thickness of 4 mm for brain tumour tissues, while normal brain tissues were adjusted. The tissues were processed consecutively in an automated tissue processor as follows:

a. 80% ethanol for 1 h
b. 95% ethanol for 1 h
c. 95% ethanol for 1 h
d. Absolute ethanol for 1 h
e. Absolute ethanol for 1 h
f. Absolute ethanol for 1 h

g. Xyelene for 1 h
h. Xyelene for 1 h
i. Xyelene for 1 h
j. Warmed paraffin for 2 h
k. Warmed paraffin for 2 h

The paraffin embedded tissue blocks were trimmed and sectioned with a microtome to obtain a thickness of 3 µm of the tissue section for immunofluorescence staining. The ribbons of sectioned tissue were floated in a 38^0C water bath and 'fished' onto a slide. The slides were put on a 60^0C hot plate or slide warmer.

Procedure for Histological Assessment

The tissue was deparaffinised with two changes of xylene, followed by xylene 1:1 with ethanol and rehydration with two changes of absolute, 95% and 70% ethanol. The tissue was then rinsed under running cold water from a tap. The flow of staining is shown below:

a. Xylene for 3 min
b. Xylene for 3 min
c. Xylene 1:1 with ethanol for 3 min
d. Absolute ethanol for 3 min
e. Absolute ethanol for 3 min
f. 95% ethanol for 3 min
g. 70% ethanol for 3 min
h. Rinse with tap water for 5 min

Immunofluorescence Staining

Enough drops of 0.1% Triton X-100/PBS were added to the tissue slide for 10 min and the tissue was washed three times with 1X PBS. A unit of 0.5% PBS/BSA was added to the tissue slide for 5 min. The tissue sections were then stained with PE-conjugated anti-human CD133 (1:15 dilution) and FITC-conjugated anti-human VEGFR-2 (1:200 dilution) to identify the tissue resident EPCs. The tissue sections were incubated overnight at 4 °C in a dark, humid incubation chamber. After the incubation, the tissue sections were washed three times with 1x PBS and counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI) for 30 min. Finally, the slides were washed three times with 1X PBS and mounted in Prolong antifade mounting reagent from Life Technologies and assessed using BX41 Olympus microscopy at 200X magnification. Excitation in the ultraviolet (330-385 nm), blue (460-490 nm) and green (510-550 nm) is used in this BX41 Olympus microscope. The ultraviolet filter was used to identify the cell nuclei stained *blue* with *DAPI,* the blue filter used to detect the FITC-VEGFR2+ marker that is reflected in green and the green filter for the PE-CD133 marker in red. Both the reflected light and sample fluorescence are viewed through and recorded at the microscope. A Nikon Coolpix 5.1 megapixel camera as well as a USB camera with image capture software were used to record the images. The images were captured and merged to review the expression of the markers.

Immunocytochemical Scoring

About 24 field images were captured of the brain-tumour sample and another 10 to 24 images of the adjacent normal brain per patient. Therefore, the total area assessed of both the tumour and adjacent normal brain for 22 patients was about 606.50 mm² for tissue resident EPCs. The images were captured at 200X magnification. The counts were expressed as the average of all fields examined. The percentage of EPCs in the tissues was analysed using Image J software version 1.45 s.

Statistical Analysis

The Mann-Whitney test was applied for comparing the independent samples and the Wilcoxon Signed-Rank test was applied for comparing related samples. The Spearmann-ranked correlation coefficient was applied to determine correlation. Statistical significance was determined at $p<0.05$ and SPSS software version 22.0 was used in the study.

RESULTS

A total of 22 astrocytic glioma patients were included in this study. The clinical characteristics of the patients are displayed in Table 1. Patients with various types of glioma were enrolled in the study; the types included pilocytic astrocytoma, diffuse fibrillary astrocytoma, diffuse astrocytoma, anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic ependymoma, anaplastic gemistocytic astrocytoma, glioblastoma with oligodendroglioma component, gliosarcoma and glioblastoma multiformae.

Table 1
Clinical characteristics of respondents

Characteristics	n (%)
Age	
≤30 years	2 (9.1)
31-40 years	6 (27.3)
41-50 years	7 (31.8)
51-60 years	5 (22.7)
>60 years	2 (9.1)
Gender	
Male	16 (70.0)
Female	6 (30.0)
Astrocytic glioma diagnosis	
Glioblastoma multiformae WHO grade IV	9 (40.9)
Anaplastic WHO grade III	8 (36.4)
Diffuse WHO grade II	2 (09.1)
Pilocytic WHO grade I	3 (13.6)

The age of the patients increased by disease severity in the following order: Grade I: 21.33±20.79 years < Grade II: 46.50±0.707 years < Grade III: 47.38±11.95 years < Grade IV: 48.44 ±10.66 years). The EPCs in the brain tumour correlated significantly with the age of the patients with positive association (Spearman's rho correlation test, r=0.52; p=0.013; Figure 1). No correlation was found in adjacent normal brain tissue with the age of the patients (Spearman's rho correlation test, r=- 0.051; p=0.820).

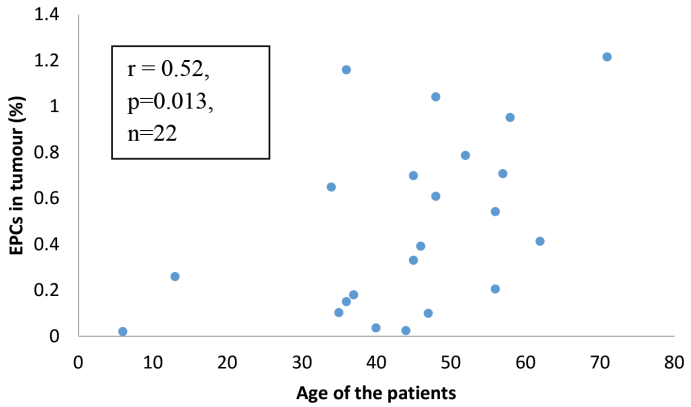

Figure 1. Correlation between tissue resident EPCs in brain tumour and age of the patients (Spearman's rho correlation test, r=0.52; p=0.013, n=22)

The tissue resident EPCs were significantly higher in the brain tumour (median=0.40, IqR=0.59) compared to the adjacent normal brain (median=0.067, IqR=0.29) (Wilcoxon Signed-Rank Test, Z stat=-3.587, p<0.001); Table II. The higher tissue resident EPCs found in Grade III and IV gliomas were compared with the low-grade (Grade I & Grade II) gliomas; however, the p-value was not significant (median=0.61, IqR=0.70 vs. median=0.26, IqR=0.30; z=-1.763 p=0.078; see Table 3). The detection of tissue resident EPCs $CD133^{+}/VEGFR2^{+}$ in the brain tumours by grade of astrocytic glioma is shown in Figure 2.

Table 2
Comparison of median epcs in the brain tumour and normal adjacent brain of patients

	Median (IqR)		Z stat[a]	p-value
	Brain Tumour	Normal Adjacent Brain		
Median EPCs	0.40 (0.59)	0.067 (0.29)	-3.587	p<0.001

[a]Wilcoxon Signed-Rank Test

Table 3
Comparison of median epcs in high-grade and low-grade glioma patients

	Median (IqR)		Z stat[a]	p-value
	High Grade	Low Grade		
EPCs	0.61 (0.70)	0.26 (0.30)	-1.763	0.078

[a]Mann-Whitney Test

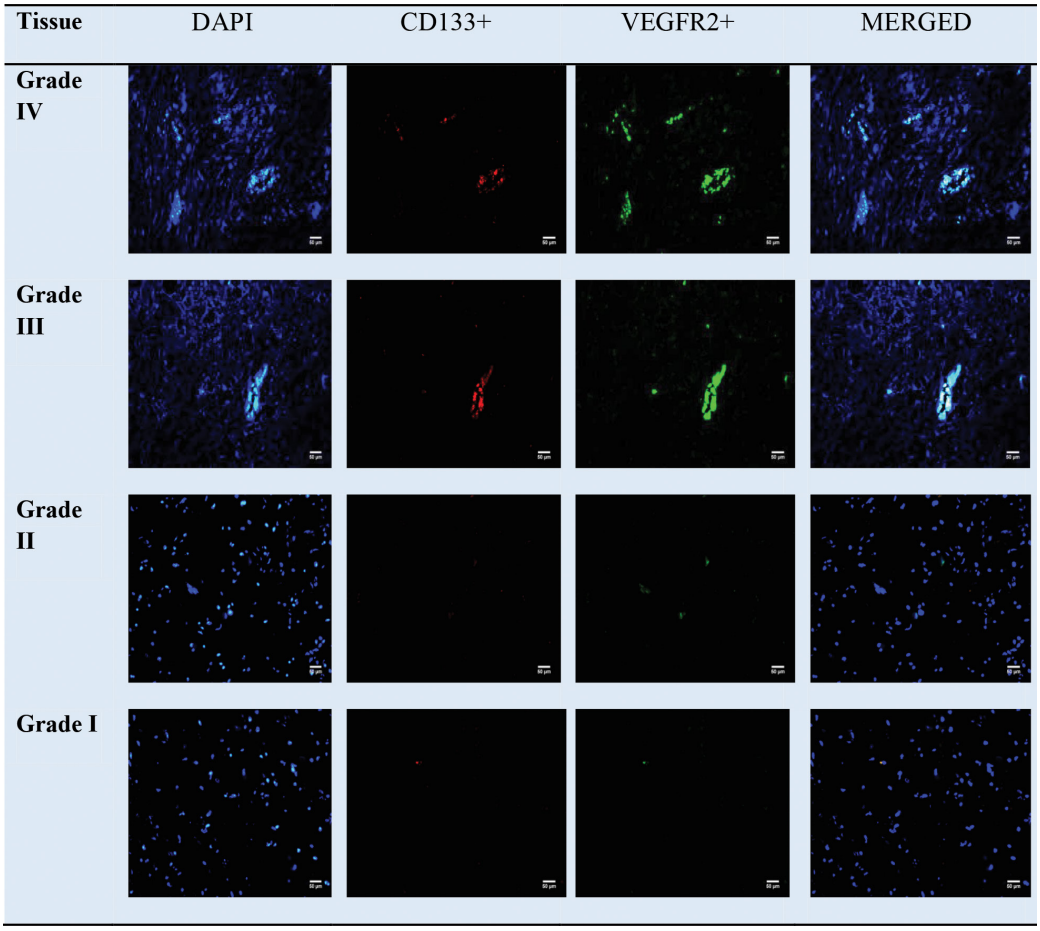

Figure 2. Detection of endothelial progenitor cells (EPCs) in tissue glioma of the patient

DISCUSSION

The World Health Organization classified astrocytic tumours in four stages. Stage I includes: Subependymal giant cell astrocytoma and Pilocytic astrocytoma; Stage II: Pilomyxoid astrocytoma, Diffuse astrocytoma and Pleomorphic xanthoastrocytoma; Stage III: Anaplastic

astrocytoma; and Stage IV: Glioblastoma, Giant cell gliobastoma and Gliosarcoma (Louis et al., 2007). Gliomas are classified as high (III and IV) and low (I and II) grade. The high-grade gliomas have more characteristics of anaplastics. They also contain immature astrocytes, oligodendrocytes or both. The low-grade gliomas are characterised by well differentiated astrocytes or oligodendrocytes lineage and are commonly diagnosed in younger patients. They have the ability to progress into anaplastic gliomas or secondary glioblastomas. However, the high-grade gliomas are mostly detected *de novo* among older patients, apart from clinically identified history (Rebetz et al., 2008). In another study it was also proven that patients with advanced age are more prone to be diagnosed with brain tumour (Zainal et al., 2006). A similar result was found in the current study, where it was found that Grade IV gliomas were diagnosed among the older patients and the less severe Grade I gliomas were identified among the younger patients.

EPC were characterised using two markers, CD133 and VEGFR-2 (KDR), that were found to be localised in structure of the capillary of the solid tumour and they promoted vasculogenesis. Microvascular density was assessed using the CD31 marker and it was found to be correlated significantly with the tumour grade as it was found to be the highest in Grade III tumour patients. CD31 is expressed on activated and non-activated endothelial cells (Hilbe et al., 2004). Previous research findings reported that the endothelial cells present most in anaplastic astrocytomas compared to low-grade astrocytomas and glioblastomas (Strik et al., 2001). Moreover, comparing the glioblastoma and low grade astrocytoma, it was found that the glioblastoma multiformae had more expression of thymidine phosphorylase, which is responsible for secreting the vascular endothelial growth factor for angiogenesis in the tumour (Yao et al., 2001), and this might explain why the higher-grade gliomas were found to have more tissue resident EPCs compared with the lower-grade gliomas in the current research findings.

The morphology of diffuse astrocytomas showed well differentiated neoplastic astrocytes in a microcystic tumour matrix, having moderate cellularity and nuclear atypia but without mitotic activity. The anaplastic astrocytomas showed diffuse increase in cellularity and nuclear atypia with presence of mitotic activity. The glioblastomas, on the other hand, were poorly differentiated, highly anaplastic and pleomorphic tumours with increased nuclear atypia and high mitotic activity. There were also prominent vascular proliferations and necrosis as well as parenchymal invasion in the anaplastic astrocytomas. The proliferation rate of the tumour determined using the Ki-67 marker was noted to be significantly increased when the low-grade (Grade II) and high-grade tumours (Grade III and Grade IV) were compared; however, no significant increment was seen between Grade III and Grade IV tumours (Stanca et al., 2012). Significant increment in the Ki67 expression with the increase in the age of the patients was also found in another study and an association between nestin and the Ki67 proliferation marker with grade of the tumours were found (Osama et al., 2010). These findings support the current research, which has reported increased EPCs in gliomas associated with the increase in the grade of malignancy and the age of the patients.

The population of endothelial cells, astrocytes, neural stem/progenitor cells, mesenchymal stem cells, pericytes and microglia/macrophage were abundantly present in the glioblastomas compared with in the normal brain parenchyma (Golebiewska et al., 2013). Malignant gliomas are known to infiltrate into adjacent normal healthy brain tissue, and this finding has been

seen in animal models using BT4C malignant gliomas. BT4C gliomas are known to be highly angiogenic as the tumour vessels metastasise to distant parts of the brain after anti-angiogenic treatment (Wirth, 2012). This finding supported that of the current study, which found that tissue resident EPCs were significantly higher in the brain tumour compared with in the adjacent normal brain.

Patients with Grade IV and Grade III gliomas who had undergone treatment (radiotherapy or chemotherapy) showed a significant reduction in circulating EPCs compared with in the healthy control. The number of EPCs in patients not undergoing treatment for Grade IV and Grade III gliomas had higher EPCs compared with the healthy control. Patients before chemotherapy and after surgery showed increment in the number of EPCs compared with the healthy patients. Chemotherapy decreases VEGF levels significantly in patients (Corsini et al., 2012). Therefore, chemotherapy based on tumour grade and age of the patient is important, and might serve as a potential indicator for improved and effective antiangiogenic treatment for gliomas.

LIMITATION

Determining the EPCs especially in the astrocytic glioma patients was a challenging task since EPCs are rare cells. Therefore, this factor should be considered when interpreting the current results.

CONCLUSION

We observed in this study that EPCs increased with age and grade of malignancy in astrocytic glioma patients. Therefore, our findings suggest that the antiangiogenic drugs given based on age and grade of tumour might be useful for preventing tumour growth in glioma patients.

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