ASSOCIATION OF TUMOR ANGIOGENIC CELLS (CD133+/VEGFA+) AND CIRCULATING CANCER STEM CELLS (CD133+/VEGFR2-) IN ASTROCYTIC GLIOMA PATIENTS

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Abstract

Background: Astrocytic gliomas are the most common primary brain tumors that developed from glial origin. The angiogenic cell population from brain tumor enhances the recruitment of circulating cancer stem cells homing towards tumor site.

Objectives: This study aimed to investigate the tumor angiogenic cell population that stained with CD133+ and VEGFA+ markers and its association with circulating cancer stem cell (CD133+/VEGFR2-) population in the peripheral blood mononuclear cells (PBMCs) of astrocytic glioma patients.

Methods: A total of 22 astrocytic glioma patients from Hospital Universiti Sains Malaysia who consented to the study were included. Tumors (n=22) were sliced and stained with CD133+ and VEGFA+ angiogenic markers and counter stained with DAPI. The circulating cancer stem cells (CD133+/VEGFR2-) in PBMCs (n=22) were quantified using FACS based on the expression of CD133 and VEGFR2 markers. The paired *t*-test and Pearson correlation were used for the data analysis.

Results: The percentage of angiogenic cell population was significantly higher in brain tumor compared to adjacent normal brain tissue (1.25 ± 0.96% vs. 0.74 ± 0.68%; paired *t*-test=2.855; df=21, p = 0.009). Positive correlation was found between the angiogenic cells of brain tumor tissue and adjacent normal brain tissue (Pearson correlation, r = 0.53, p = 0.011). Significant positive correlation was found between angiogenic cells in glioma tumor and cancer stem cells in peripheral circulating systems of astrocytic glioma patients (Pearson correlation, r = 0.42, p = 0.049).

Conclusion: Angiogenic cells in the brain tumor resident promote the recruitment of circulating cancer stem cells homing to the tumor site and induce the proliferation and growth of the tumor in astrocytic glioma patients.

Keywords: *Glioma, Astrocytic, Astrocytomas, Angiogenic Cells, Brain Tumors*

Introduction

Organ-specific pluripotent and committed stem cells are found in the bone marrow as a rich reservoir. Number of cells stored in the bone marrow such as hematopoietic, endothelial, and mesenchymal stem cells are being transported into the peripheral circulating systems (1). It is believed that the glioblastoma arises from the stem-

like cancer cells with high expression of the CD133 gene known as Prominin1, transmembrane protein. Comparison between mice that was co-transplanted with Prom1$^-$ endothelium and mice that was co-transplanted with proneural tumor sphere cells and Prom1$^+$ endothelium showed that the latter had a significantly more tumor burden and a higher vascular sprouting or angiogenesis. Prominin-1 is capable of tumor initiation of VEGFR2-GFP. Prom1$^+$endothelium induced tumors were found to have increased in size, have more distorted vessels, thickened walls and a higher tumor cell density which enhance its function in promoting tumor growth (2).

Primary tumor secretes vascular endothelial growth factor (VEGF) that is responsible for the proliferation and migration of the tumor. The binding of VEGF to its respective receptor on the endothelial cells initiate the angiogenesis and mitogenic signal. In a previous study it has been reported that the radiation induced VEGF in cultured medium showed that the irradiated glioma cells have strong tumor motility because the VEGF phosphorylates VEGFR2 and this promotes VEGFR2-mediate downstream signalling. The signal then activates the Src and FAK phosphorylation promoting glioblastoma multiformae tumor cells migration and cell invasion. Furthermore, the blocking of VEGF by anti-VEGF antibodies showed low tumor migration and cell invasion and it is necessary for better treatment (3).

Prominin-1/CD133 is used as cancer stem cells surface marker. The marker is classified as pentaspan transmembrane protein which regulates angiogenesis, apoptosis and shows effect on the vascular endothelial growth factor (VEGF). In a previous study of melanoma cell lines, the knocking down of prominin-1 in the cells reduce the binding of VEGF and show more apoptosis. Prominin-1 may hold significant potential target of cancer therapy since Prominin-1 expression enhances the proliferation and cell survival with direct correlation. The study found that prominin-1 is essential for the growth of melanoma cells (4). A previous study on astrocytomas that were embedded in formalin fixed paraffin, also found localization and distribution of CD133 marker which is defined as a putative brain tumour stem cell marker. It was found that the tumour expresses CD133 in all grades of tumors (5).

It has been hypothesized that tumor stem cells in the gliomas or astrocytomas originated from neural stem cells or by attracting the neural stem cells towards the tumor site (6). High secretions of VEGF were found in tumor with high fraction of cancer stem cells cultures. Therefore, targeting therapies of cancer stem cells to cytotoxic or antiangiogenic combination regimens might eradicate the proangiogenic signalling of these cells (7). Therefore the current study is aimed to investigate the association of tumor angiogenic cells that expresses (CD133+/VEGFA+) markers that may induce the mobility of circulating cancer stem cells (CD133 +/VEGFR2-) towards the tumor site to enhance the proliferation of tumor growth in astrocytic glioma.

Materials and Methods

Study Participant

Astrocytic glioma patients (WHO grade I to IV) from the Universiti Sains Malaysia Hospital (HUSM) were recruited in the study. The sampling was done over a 3-year period (2012–2014). Twenty-two patients with confirmed diagnosis of astrocytic gliomas with histological confirmation were enrolled 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). All astrocytic glioma patients who consented to the study were included.

Blood Sampling

The peripheral venous blood was obtained during surgery from each patient for the analyses of circulating cancer stem cells. Twenty-five ml of peripheral venous blood was collected in EDTA tube via venipuncture from each patient at the time of surgery. To minimize contamination with endothelial cells from the puncture wound of the vascular wall, the initial 5 ml blood sample drawn was discarded (8). The blood samples were processed within 1 hour after collection.

Flow Cytometry Studies

Measurement of counts of circulating cancer stem cells in peripheral blood is quantitatively determined using FACSCanto II flow cytometer (Becton Dickinson, USA). The peripheral blood mononuclear cells (PBMCs) were prepared by gradient centrifugation using Ficoll-Hypaque technique. After the preparation, about 10^6 cells of PBMC cells were pipetted into separate 1.5 ml centrifuge tubes and 10 µl of FcR-blocking reagent was added for 10 minutes to inhibit non-specific bindings. Then the cells were incubated with 10 µl of phycoerythrin-conjugated (PE) anti-human CD133 monoclonal antibody (mAb) (Miltenyi Biotech), 10 µl of allophycocyanin-conjugated (APC) anti-human VEGFR-2 mAb (Miltenyi Biotech) and FITC annexin (Miltenyi Biotech) for 30 minutes at 4°C. The PE-, APC- and FITC- isotype-matched IgG1 (Miltenyi Biotech) antibodies were used as a measurement of negative controls. Any unbound antibodies were removed by washing 3X with phosphate buffered saline (PBS). Finally, the cells were re-suspended in 400 µl of FACS solution and analysed with FLowJo_V10 software. About 50 000 events were collected in the FACS analysis of each probe that were performed in duplicate. The frequencies of circulating cancer stem cells in peripheral blood were determined by 2D side-scatter/fluorescence dot-plot analysis of the samples. Circulating cancer stem cells are expressed as percentage of total PBMCs for each patient (9).

Tissue sampling

Microsurgical specimens of brain tumor and adjacent normal brain tissue from the same 22 astrocytic glioma

patients were obtained from each patient after the surgery for the analyses of tissue resident angiogenic cells CD133+/VEGFA+. Tissue biopsy was fixed in 10% paraformaldehyde and analysed for immunofluorescence microscopy.

Immunofluorescence Microscopy

The percentage of CD133+VEGFA+ in the tumor specimens was characterized using the immunofluorescence microscopy method. Tissue biopsy was fixed in paraformaldehyde 10 %. Brain tissue samples and adjacent normal brain tissue each with 3 sections were cut at an interval of 6.5 mm with a thickness of 4 mm. The tissue was then processed in a tissue processor and embedded in a paraffin block. Tissue blocks were cut in 8 serial dissections with a thickness of 3 µm. The tissue then was deparaffinized with 2 changes of xylene, then with xylene and ethanol, 1:1 and rehydration with 2 changes of absolute, 95% and 70% ethanol, 3 minutes each respectively. Then the tissue was rinsed under running cold tap water. The tissue was applied with drops of 0.1% Triton x-100/PBS for 10 minutes and washed 3 times with 1X PBS. Finally, the tissue was added with PBS/BSA 0.5% for 5 minutes.

The tissue sections were then stained with two antibodies; phycoerythrin-conjugated (PE) anti-human CD133 monoclonal antibody (mAb) and fluorescein isothiocyanate (FITC) anti-human VEGF (Bioss) and were left overnight in a dark humid incubation chamber at a temperature of 4°C. The tissue then was washed 3X with PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 30 minutes. The slides were washed 3X with PBS and mounted in prolong antifade (life technologies) mounting medium and assessed through BX41 Leica microscopy. For each patient a total of 24 fields image were captured for brain tumour sample and another 24 fields for adjacent normal brain. Therefore, the total area assessed for 22 patients for both tumour and adjacent normal brain were 606.50 mm^2. The images were captured at 200X magnification. The counts were expressed as the average of all fields examined. The images were captured at 200X magnification and were merged to find the co-localization of the 2 markers. For each sample of brain tumor about 13784 mm^2 of the tissue area was investigated. The percentages of angiogenic cells in the tumor and adjacent normal brain (CD133+VEGFA+) were determined using image J software 1.45s.

Statistical Analysis

The paired *t*-test was performed. Correlation analyses (Pearson correlation) were performed based on normality of numerical variables. Statistical significance was set at $p < 0.05$. All analyses were performed using SPSS software version 20.0.

Results

Participant Characteristics

Twenty-two patients with astrocytic glioma were recruited in this study which included all grades of glioma [WHO grade IV (n=9), grade III (n=8), grade II (n=2), grade I (n=3)]. Types of diagnosis were pilocytic astrocytoma, diffuse fibrillary astrocytoma, diffuse astrocytoma, anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic ependymoma, anaplastic gemistocytic astrocytoma, glioblastoma with oligodendroglioma component, gliosarcoma and glioblastoma multiformae. Socio-demographic and clinical characteristics of patients are shown in Table 1. All data are presented as mean ± SDs.

Table 1: Socio-demographic and clinical characteristics of participants

Characteristics	n (%)
Age	
≤40 years **>40 years**	8 (40.0) 14 (60.0)
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 (9.1)
Pilocytic WHO grade I	3 (13.6)

To define angiogenic cell population, the tumor brain tissue and normal adjacent tissue of glioma were stained with two antibodies using CD133+/VEGFA+ markers to find newly formed blood vessels. The immunofluorescence staining of co-localisation of the marker of brain tumor tissue resident and adjacent normal brain were shown in Figure 1. The angiogenic cells in brain tumor tissue were higher than adjacent normal brain tissue (1.25 ± 0.96% vs. 0.74 ± 0.68%; paired *t*-test=2.855; df=21, p = 0.009). Positive correlation was found between the angiogenic cells of brain tumor tissue and adjacent normal brain tissue (Pearson correlation, r = 0.53, p = 0.011) (Figure 2).

In circulating systems of astrocytic glioma patients, we used CD133+/VEGFR2- markers to define the circulating cancer stem cells. Stringent gating systems was utilised in the flow cytometry analyses to find the percentage of PBMCs population that stained with CD133+ and VEGFR2- markers as shown in Figure 3.

Significant positive correlation was found between angiogenic cells (CD133+/VEGFA+) in brain tumor and circulating cancer stem cells (CD133+/VEGFR2-) cells in astrocytic glioma patients (Pearson correlation, r = 0.42, p = 0.049) (Figure 4). However, no significant correlation was found between adjacent normal brain tissue and circulating cancer stem cells (Pearson correlation, r = -0.12, p = 0.583).

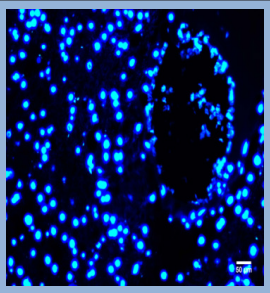
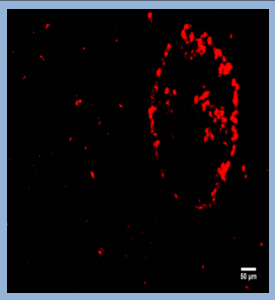
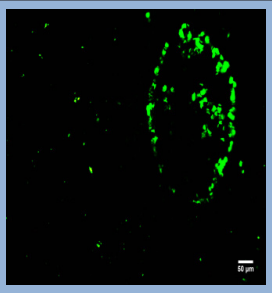
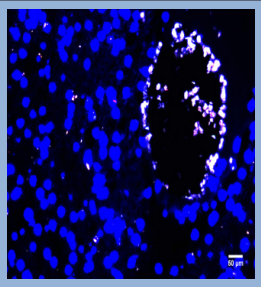
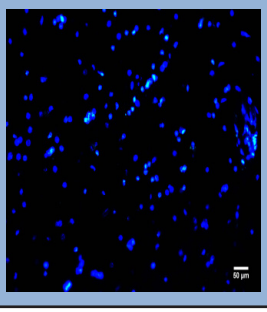
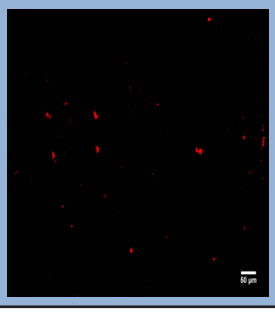
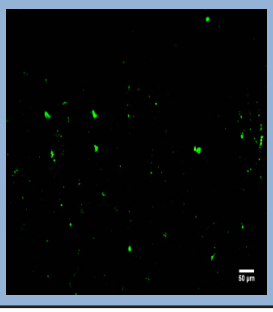
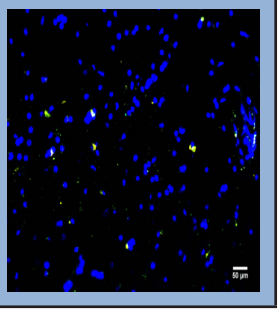
Tissue	DAPI	CD133+	VEGFA+	MERGED
Brain Tumor				
Adjacent Normal Brain				

Figure 1: Immunofluorescence staining demonstrate the presence of tissue-resident CD133+/ VEGFA+ population in astrocytic glioma

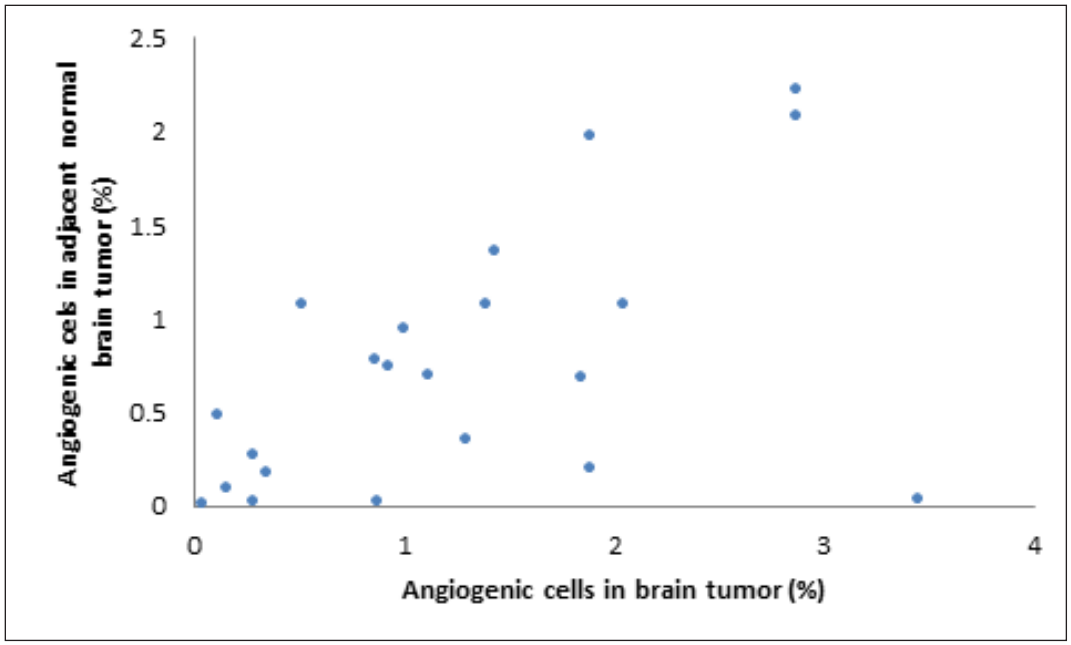


Figure 2: Correlation between angiogenic cells (CD133+/VEGFA+) in brain tumor and normal adjacent brain of astrocytic glioma patients

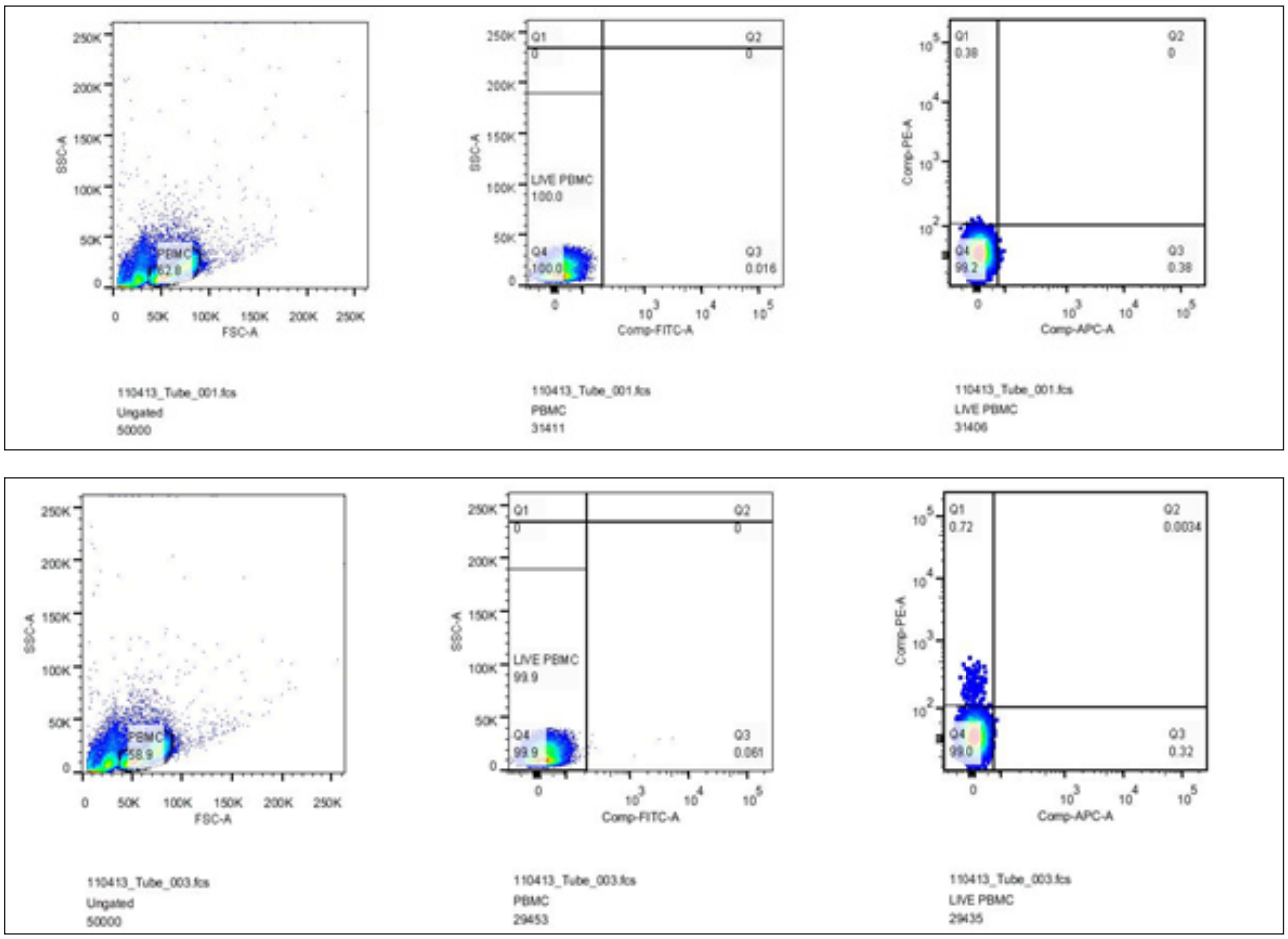


Figure 3: Flow cytometric analysis demonstrate the presence of circulating cancer stem cells that were stained with CD133+/VEGFR2- markers

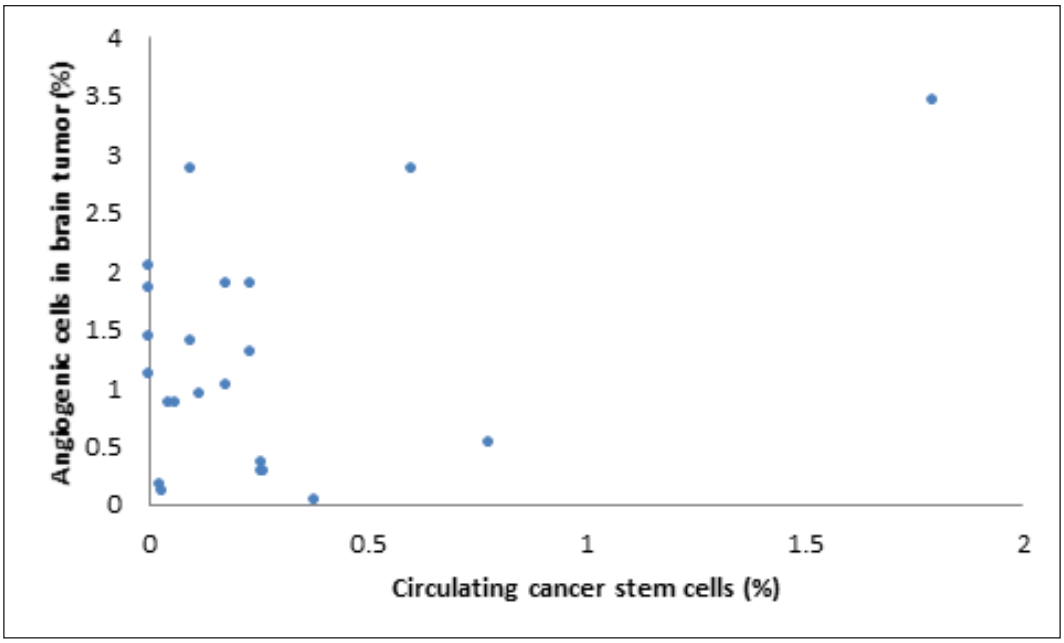


Figure 4: Correlation between angiogenic cells (CD133+/VEGFA+) in brain tumor and circulating cancer stem cells of astrocytic glioma patients

Discussion

The current study found a significant positive association between angiogenic cells in brain tumor and circulating cancer stem cells of astrocytic glioma patients. This is supported by the previous finding that indicates glioblastoma multiformae grows from the CD133+ cell population, thus CD133 generates tumour cells and also its endothelium. Increased infiltrative and hyperproliferative tumors can be found in double positive CD144+/CD133+ and single positive CD133+/CD144- population with more expression of nestin. The CD133+ found in brain tumor stem cells and has the capability in the formation of tumors with more vascularization. Tumorigenicity was tested by single sorted CD133+ which was characterized under self-renewal and angiogenic cell fraction, dual sorted $CD133^{+}$/$CD34^{-}$ as self-renewal cell fraction and single sorted $CD133^{-}$ represents the absence of self-renewal and angiogenic cell fractions. The results showed that the mice implanted with CD133+ tumor cells showed bigger and more vascularized tumor compared to tumors implanted with $CD133^{-}$ tumor cells (10).

The cancer cells and cancer stem cells which express Nestin+/CD133+ were found to be localized next to the capillaries of brain tumors. Interestingly cancer stem cells that were found at the vascular niche of the tumor undergo self-renewal and differentiation by the help of endothelial cells. These Nestin+/CD133+ cancer stem cells signal the endothelial cells to secrete compounds for the cancer cells renewal and facilitate more growth of tumors and also to maintain the undifferentiating conditions. The tumor cancer stem cells fraction with CD133+ expressions, cultured with the primary human endothelial cells indicates that the endothelial cells accelerate the proliferation of the tumor. On the other hand, depletion of the brain tumor blood vessels eradicates the properties of self-renewal of the tumor cells. Thus, the antiangiogenic which targets on the endothelial cells may be useful to prevent the tumor growth by distrupting the microenvironment of the tumor vascular niche. It has been speculated that the endothelial cells may promote tumor radioresistance. One of the best treatments such as VEGF-specific inhibitors provide useful tools to eradicate the tumor growth since it targets on the vascular niche of the tumor environment and is associated with the cancer stem cells (11).

In a previous study, researchers have found the relationship between cancer stem like cells of glioblastoma multiformae and endothelial cells that promote the tumour growth. The mechanism involved was the NOTCH ligands of endothelial cells that bind to NOTCH receptors of the glioblastoma multiformae cancer stem like cells and enhance the self-renewal of the cancer stem like cells. It was found that the Nestin-postive tumor was colocalized with the NOTCH receptor in the primary glioblastoma multiformae (GBM) and it has increased the activity of the NOTCH level by the ligand JAG1- or DLL1 expressing cells such as endothelial cells which are located next to the NOTCH receptors. This pathway activation is induced by HES5. Tumor cells also were found with JAG1-ligand expressing cells. Thus, the human brain microvascular endothelial cells within GBM may play a role as a cancer stem like cell niche by NOTCH ligands to NOTCH receptors that were expressed by the cancer stem like cells GBM population. The study also found that the differentiated GBM tends to lose the level of NOTCH activity and reduced CD133 population and become less tumorigenic. The cancer stem like cells marker of CD133 and CD15 that were induced in JAG1 or DLL1 peptide-treated GBM neurospheres might promote the activation of NOTCH signaling by the ligand and enhance the self-renewal of GBM. The human brain microvascular endothelial cells stimulate GBM cancer stem like cells self-renewal since CD133 marker was increased significantly. The neurosphere of GBM was increased in human brain endothelial cells culture than the culture with the medium only and the knockdown of JAG1 by shRNA expression in these endothelial cells have reduced the cd133 expressing cells and cd133 mRNA expression. The abrogated growth of the GBM in intracranial xenografts was found because the cancer stem like cells self-renewal were reduced. Therefore, the treatment should be aimed at interrupting the relationship or interaction of NOTCH signalling by NOTCH ligand blocking antibody or peptide, in combination with chemo- and radiation-therapy that may provide novel therapeutic treatment for the patients. The study also speculated that the NOTCH pathway activations may originate from VEGF that stimulates the DLL4 ligand and also the nitric oxide (NO) that is released from tumor endothelium diffuses to neighboring glioma stem-like cells responsible for the GBM growth (12). This supports the current research finding that found (CD133+/VEGFA+) tumor angiogenic cells correlate with the circulating cancer stem cells (CD133+/VEGFR2-).

In another study it has been found that the glioma stem cells secrete VEGF due to the hypoxia condition and this VEGF level is much more elevated compared to non-stem cancer cells. The glioma stem cells upregulate the hypoxia-inducible factor HIF2A mRNA and this HIF2a coexpressed together with some CD133+ cells. Therefore, the study hypothesized that the HIF2a is essential for the growth and survival of glioma stem cells. HIF 1a and HIF 2a knockdown under hypoxia suppress the secretion of VEGF protein and its promoter activity intracellular and exhibit distorted tumor formation. The survival rate for the glioma patients seems to be associated with these elevated HIF2a expression. Moreover, the HIF2a positive cells were located adjacent to the blood vessels which was indicated by the immunohistochemical analysis. The study has also found that HIF1a is important as a tool for the proliferation, survival, and angiogenesis for cancer stem cells and non-stem cancer cells. Targeting the HIF and VEGF coexpression might serve as a better therapeutic agent for the glioma cases (13).

Cultured cells in the stem cell medium that contained CD133+ glioma cells, formed neurosphere-like spheroids that were taken from the biopsy and xenografts and identified as stem cell like glioma cells. It was found that the cells were capable to, transdiffrentiate into neuronal,

astrocytic and oligodendrocytic cells. The CD133+ glioma population was also found with the properties of increased tumor vascularity, necrosis, and hemorrhage. In xenograft samples of mice, it was found that the brain that was implanted with CD133+ tumor cells exhibited high large angiogenic, proliferative and vascular tumors with more necrosis and hemorrhage tumors compared to brains implanted with CD133- tumor cells. Both patient biopsies and human glioma xenografts indicated that the VEGF levels were up-regulated as much as 10 to 20 folds in the media of CD133+ glioma cell than CD133-. The level of VEGF was measured through the ELISA and it was found that the hypoxia condition, induced VEGF secretion in both the CD133+ and CD133- glioma cells. However, the VEGF secretions in CD133+ were found to be more than the CD133- glioma cells. The stem cell like glioma cells also have the potential to form tube by increasing the length of human microvascular endothelial cell tube number of branch point, and tube complexity and stimulates the endothelial cell migration. The authors also suggest that the CD133+ cells influence endothelial cell behavior through proangiogenic factors (14).

The treatment that involves inhibition of VEGF were developed such as anti-VEGF antibody, bevacizumab/ Avastin. This antibody reacts by binding to the human VEGFA ligand to prevent endothelial cell receptor activation by blocking the proangiogenic effects on endothelial cell migration and tube formation. Therefore, stem cell like glioma cells on endothelial cell behavior are dependent on VEGF activity. In the xenografts models the growth of stem cell like glioma cells showed reduction of tumor weight, vascularity, and hemorrhage after blocking with anti-VEGF antibody. Even though stem-like cells in gliomas are found to be less, it may be significant for tumor growth through self-replication and paracrine effect for non-stem-like tumor cells as the bevacizumab does not significantly inhibit tumor growth but inducing neovascularization in non-stem like tumor cells. Thus, the results suggest that only a subpopulation such as of the tumor driving VEGF mediated tumor angiogenesis may be affective for the VEGF neutralizing antibody (bevacizumab). The CD133+ tumor cells were detected at proximal locations to blood vessels. It was found that the glioma stem like cells do not express VEGF receptors thus, targeting VEGF alone may not be effective on cancer stem cells but might be effective on paracrine effects on endothelial cells and possibly other cancer cells (14).

In another study the tumours that mimic the glioblastomas were rapidly formed when adult rat white matter progenitors infected with Platelet-Derived Growth Factor (PDGF-B)-expressing retrovirus. The uninfected progenitor cells were also recruited and stimulated to proliferate through this paracrine growth factor stimulation. The study found that the glial progenitors have a tendency to express proliferative and self-renewing characteristics to form tumours. The tumour grows by diffusely infiltrating the brain and intermingles with normal brain cells and its environment through autocrine and paracrine signaling. This could be explained by the possibilities of non-transformed glial progenitors that were entrapped within an infiltrating glioma that might be induced to proliferate by signaling of paracrine PDGF (15). Therefore, in the current study the positive association between angiogenic cells in the tumor and adjacent normal brain tissue suggest that tumor angiogenic cells may enhance the tumour growth.

Conclusion

We observed correlations between the number of circulating cancer stem cells and tissue angiogenic cells in brain tumor. Circulating cancer stem cells in the peripheral blood of astrocytic glioma patient are positively correlated with brain tumor however no correlation was observed with normal adjacent brain tumor. Thus, targeting tissue resident angiogenic cells and cancer stem cells by antiangiogenic treatment may be useful in treatment of astrocytic glioma.

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Competing Interests

The authors declare that they have no competing interests.

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