

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Blood-Brain Barrier Breakdown by Combined Detection of Circulating Tumor and Endothelial Cells in Liquid Biopsy

Natalia Malara and Giuseppe Donato

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.80594>

Abstract

Blood-brain barrier breakdown occurring in glioblastoma is a temporary condition often denounced by contrast enhancement upon neurological examination. This condition is useful to increase the intracranial concentration of anti-cancer drugs. The prognosis of glioblastoma and its resistance to conventional therapy has stimulated interest in the search of biomarkers able to unmask and monitor brain barrier breakdown to calibrate the treatment. Despite numerous studies had evidenced the role of circulating tumor and endothelial cells to monitor brain tumor, the mechanism of tumor cells release in the bloodstream and its prognostic significance remain unclear. In this chapter, we want to furnish an update on the relationship between the vascular damage occurring during glioblastoma disease and the reactivity of innate immunity focusing on the cytokines network. Our aim is answer to the question: when and why the liquid biopsy is useful in glioblastoma disease.

Keywords: blood-brain barrier, glioblastoma, liquid biopsy, circulating tumor cells, circulating endothelial cells

1. Introduction

Gliomas are associated with aggressive invasion of the surrounding brain parenchyma. The invasion is due to a combination of transformed cells phenotype changes, innate and acquired immunity and autocrine and paracrine release of growing and permeabilizing chemokines. This intricate scenario develops within the cerebral parenchyma, notoriously protected by an ultraspecialized system. Several experimental models of glioma, have been used in vivo to

monitor the infiltration of tumor cells in the parenchyma and/or perivascular spaces, even at a single cell level [1–3]. However, the relationship between glioma-induced BBB dysregulation and glioma invasion remains poorly understood. In this chapter we review the natural history of glioblastoma and, on the basis of the scientific evidence published to date, we try to give an explanation and meaning to biomarkers found in the peripheral blood. In particular, we focus our attention on cellular biomarkers, the circulating tumor cells and cellular endothelial progenitors. Our interest is aimed at giving an order in the context of human cell biology of human glioblastoma. This intracranial tumor remains today one of the big killers and represents a major challenge in the field of oncology because it is unresponsive to treatment and able to progress in a way difficult to monitor.

2. Development and structure of the blood-brain barrier

Blood-brain barrier (BBB) separates in a selective manner the nerve tissue of the central nervous system (CNS) from the blood. It is present throughout the CNS, except in the circumventricular organs, where the capillaries are fenestrated as occurs in choroid plexuses. Indeed, such an anatomical membrane can selectively transport, through the capillary wall, large (>500 Da) or water soluble (hydrophilic) substances, whereas, small, lipid-soluble (hydrophobic) substances can freely pass the endothelium by passive diffusion [4, 5].

Classically, BBB was recognized as the structural base of the so-called *immune privilege* of the brain. Thereby, antigens would be sequestered within the brain and would be invisible to the immune system [6].

This view has been challenged in recent years, due to the discovery of the so-called “glymphatic system” (GS) and of meningeal lymphatics (MLs) of dura mater. GS allow that cerebrospinal fluid flows into brain within periarterial spaces and interstitial fluid and solute clear via perivenous spaces. Furtherly, MLs follow dural blood vessels and cranial nerves and exit the cranium via the foramina together with the venous sinuses, arteries, and cranial nerves in order to join cervical lymph nodes. The relationship of this system with the equilibrium of the blood-brain barrier is unknown and its role in human pathology has yet to be clarified (**Figure 1**) [6].

Tight junctions (TJs) between endothelial cells (ECs) are the main component of BBB. TJs are occluding cell junctions, which act to seal off the intercellular space and consist of transmembrane proteins such as occludins (Ocns) and claudins (Cldns), connected intracellularly to the actin filaments, forming strands in the plasma membrane.

TJs blocks the intercellular pathway and forms a barrier between the arterial blood and the nervous tissue, regulated by the end feet of astrocytes that cover the basement membrane of the capillaries [5].

The development of the BBB begins with angiogenesis, started from invasion of the neuroectoderm by endothelial progenitor cells from pre-existing vessels. The endothelia of these vascular sprouts show many characteristics of mature BBB such as TJs, transcytotic vesicles, nutrient transporters and leukocyte adhesion molecules. Afterwards, contact with CNS cells and pericytes (PCs) allows the full functional maturation of the membrane separating nervous

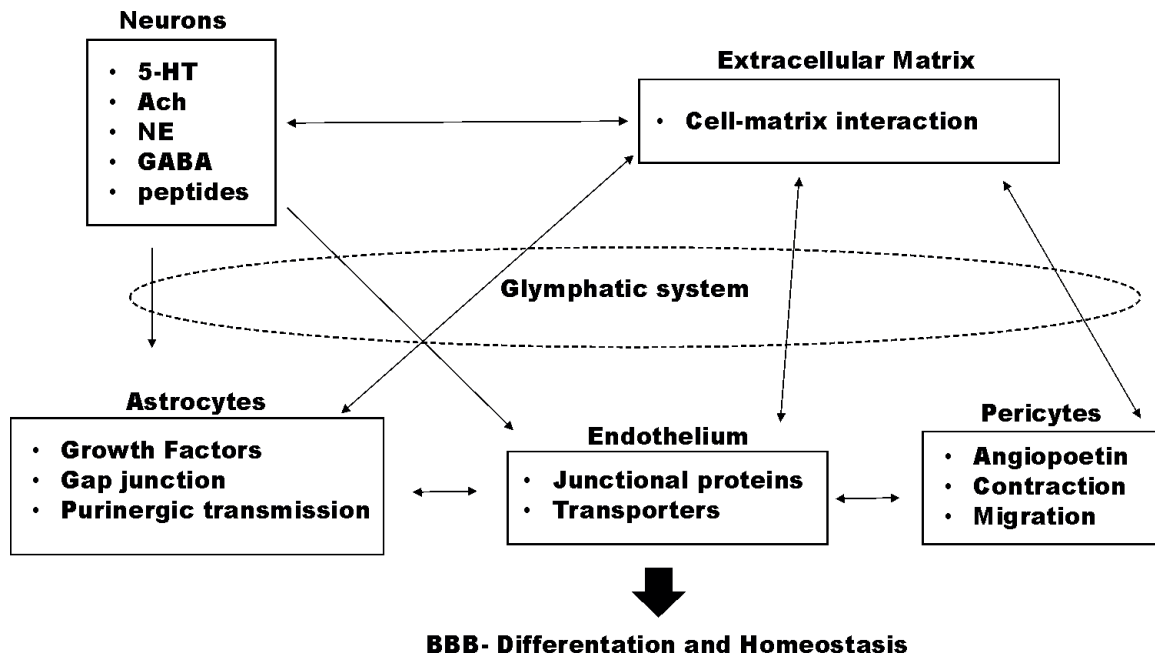


Figure 1. Blood-brain barrier components and their interactions (figure partially modified from Hawkins BT and Davis TP [105]).

tissue from blood. TJs are elaborated and sealed, transcytosis decreases, leukocyte adhesion molecules are downregulated and efflux transporter expression increases [7].

Vascular endothelial growth factor (VEGF), VEGFR-2 and its ligand, play a pivotal role in embryonic angiogenesis and vasculogenesis [8]. In the developing CNS, embryonic brain cells of the subventricular neuroectoderm synthesize VEGF that directs angiogenesis via a concentration gradient through the angiogenic sprouting from vessel networks outside the CNS, in particular, the perineural vascular plexus (PNVP). Within the brain, blood vessels furtherly, then, generate huge networks as the neural tissue grows and concomitantly remodel into a vascular tree with arterial and venous hierarchy [9, 10]. Such a process, when VEGF is reduced or absent, develops in an incorrect way generating decreased blood vessel branching and density in the cortex [11].

Vasculogenesis and angiogenesis have been extensively studied in experimental setting in mouse retina and hindbrain and in zebrafish with the development of the “tip-stalk” model of angiogenic sprouting, which describes the different precursor of endothelial cells within newly formed vascular sprouts:

1. Tip cells drive the sprouting, migrating and extending filopodia that scan the environment for signals that can act as guides for vascular growth.
2. Behind the tip cells, another group of elements, termed stalk cells, proliferate and form the nascent vascular lumen.

Once mature connections and blood flow have been established, the proliferation and the migration of “activate endothelium” ceases. Tip and stalk cells display differential gene expression profiles [12]. The Notch signaling pathway is a very important tool in the regulation

of the tip and stalk cells specification. The activation of Notch signaling inhibits tip cells differentiation and promotes the stalk cell phenotype [13].

Wingless-type mouse mammary tumor virus (MMTV) integration site family (Wnt) pathway plays a pivotal role in angiogenesis both physiological and pathological and in vessels remodeling [14, 15]. Three Wnt signaling pathways are known: the so-called “canonical” Wnt/ β -catenin and the two “noncanonical” pathways: the Wnt/calcium (Wnt/ Ca^{2+}) and the Wnt/planar cell polarity (Wnt/PCP) [16].

The canonical Wnt/ β -catenin signaling pathway targets the regulatory molecule β -catenin. The so-called cytoplasmic destruction complex, consisting of glycogen synthase kinase-3 β (GSK3 β), Axin, Casein kinase1/2 (CK1/2), Protein phosphatase 2A (PP2A), and adenomatous polyposis coli (APC) leads to a post-translational modification status of β -catenin. In the absence of Wnt signaling, cytosolic β -catenin is phosphorylated by CK1 at Thr41, and GSK3 β at Ser33 and Ser37. Phosphorylated β -catenin is then ubiquitinated via the E3 ligase, β -transducing-repeat-containing protein (β -TrCP), and thereby prepared for proteasomal degradation. Conversely, Wnt binding to Frizzled proteins, a Wnt receptors family recruits the co-receptor LRP5/6, that causes an activation of the cytoplasmic phosphoprotein Disheveled. These events lead to the inhibition of GSK3 β , thereby promoting the accumulation of unphosphorylated β -catenin and its subsequent translocation to the nucleus, where it binds to a variety of transcription factors, including T-cell factor/lymphocyte enhancing factor (TCF/LEF) and forkhead box (in particular, the FOXO subtypes) family proteins. TCF/LEF represses targets in the absence of signaling, but β -catenin, when its pathway is activated, enters into the nucleus, binds to TCF on the chromatin and allows the transcription of a number of Wnt target genes involved in cell proliferation, Wnt signal transduction and vascular growth [17, 18].

Tight junctions are organized to form a paracellular seal in transporting epithelia in order to allow the directional transfer of ions and solutes across cell layers.

Tight junctions comprise several trans-membrane proteins (TMPs) which interact with adaptor proteins of the cytoplasmic plaque via their C-terminal domains. TMPs are classified on the basis of their number of transmembrane domains (tetraspan, trispan, and single-span domains) and include the tetraspan Marvel-domain proteins (occludin, tricellulin, and MarvelD3), the claudin family of proteins, the trispan BVES (blood vessel epicardial substance) protein, the single-span JAMs (junctional adhesion molecule-A, -B, and -C), and the polarity determinant Crumbs3. The cytoplasmic plaque is composed of Zona occludens proteins (ZO-1, ZO-2, and ZO-3), multi-PDZ domain protein 1 (MUPP1), cingulin, protein associated with Lin-7 (PALS1), Pals1 associated tight junction (PATJ), protease activated receptor 3 (PAR3) and protease activated receptor 6 (PAR6), which interact in the intracellular space with the cytoskeleton. Moreover, several signaling molecules are associated to the proteins of the cytoplasmic plaque [19].

Oclns and Cldns mainly characterize TJs. The exact role of OcLn is not well known, but it may be a structural component important in the formation of TJs. Its function is regulated via cytokines, proteases and GTPases [20].

The Wnt/ β -catenin pathway, play role in BBB differentiation and may be fundamental in BBB maintenance. Such a pathway is also active in endothelial cells of the adult CNS, providing an essential tool for BBB maintenance [21]. Claudin family member claudin-5 (Cldn5) is important

for TJs formation. Cldn5 is regulated by the Wnt/ β -catenin pathway, however activation of VEGF, or other signaling pathways, can oppose the action of Wnt/ β -catenin pathway. The Embryonic ablation of Cldn5 in mice induces early postnatal brain edema and death [21]. Claudin-5-deficient mice exhibit an increased leakiness for small-molecular compounds (<800 Da) [22]. BBB leakiness can be tolerated during embryogenesis as long as the placental barrier is functional. A post-natal maturation of brain circulation is a more than likely fact. Indeed, in the mammalian brain, angiogenesis in the cortex well proceeds until 2–3 weeks after birth [23].

Interestingly, in the regions where the corners of three epithelial cells meet, TJs have a specialized structure, the so-called tricellular junctions (tTJs) which contain the tetraspan Marvel-domain protein tricellulin. Such a protein has been detected in the rat and human brain [24, 25]. Tricellular junctions may be critical for the BBB formation. Moreover, lipolysis-stimulated lipoprotein receptor (LSR), a component of paracellular junctions in their three cell membranes meeting points, expression follows CNS angiogenesis and correlates with BBB formation during embryogenesis [26].

PCs are contractile cells surrounding the endothelium of capillaries and postcapillary venules, which are enclosed within the basal lamina of the endothelium along the vessels. They behave as mesenchymal multipotent stem cells giving rise to ECs or smooth muscle cells. PCs play an important role in the angiogenesis and in the BBB integrity. Pericytes show specialized characteristics and roles in different organs such as kidney, liver and brain. Moreover, density of pericytes and vessel coverage vary among tissues [27].

A direct contact between pericytes and ECs is established where the basement membrane is absent via the “peg-and-socket” junctions which are formed by n-cadherin and connexin-43 hemichannels. Adherent junctions between PCs and ECs are also present. Interactions of ECs with PCs and SMCs are pivotal processes in the regularization, remodeling, stabilization and function of vascular wall and BBB, i.e., by the regulation of the transcellular barrier [27–29].

Moreover, the single adhesion receptor CD146 functions on PCs as a co-receptor for Platelet-derived growth factor receptors beta (PDGFR- β) to regulate interactions between ECs and PCs. CD146, shows an initial expression on ECs, during BBB maturation, that slopes down upon PCs recruitment and BBB maturation [30]. Interestingly, astrocytic laminin induces pericyte differentiation from the resting stage to the contractile stage, switching pericyte function from stabilizing the BBB to compromising it [31].

Astrocytes (ACs) surround microvessels and capillaries and interact with endothelial cells through the end-feet of their processes. ACs play critical roles in regulating cerebral blood flow in response to neuronal activity by relaying signals and maintain BBB function by inducing barrier properties and the polarization of transporters [28]. Under steady-state conditions ACs promote BBB homeostasis through soluble factors such as Sonic hedgehog (Shh), retinoic acid (RA), glial-derived neurotrophic factor (GDNF) and angiopoietin1 (Ang-1), which interact with receptors on ECs to increase junctional protein expression, raise transendothelial electrical resistance (TEER), and reduce permeability. Furthermore, knocking out a-dystrobrevin (a-DB), a scaffolding protein of the astrocytic endfeet, or astrocyte-secreted laminin a2, leads to down-regulation of junctional proteins and a leaky BBB [32]. Interactions between ACs and ECs are very important not only for formation and maintenance of BBB, but also for astrocytic differentiation [21].

Morphology and function of BBB are linked as shown by microscopic observation. Early ultrastructural studies were performed by administration of silver nitrate in the drinking water of rodents [33]. They showed the presence of very scarce quantity of silver around the capillaries in the cerebral cortex, medulla, and cerebellum. In such brain regions, capillaries are continuous. In other areas, such as neurohypophysis, area postrema, pineal body and inter-columnar tubercle, heavy accumulation of silver was present around fenestrated capillaries [34]. Using lanthanum and horseradish peroxidase as tracers, via intravenous injection, it has been demonstrated that these substances are unable to penetrate between the endothelial cells because of the tight junctions presence (zonulae occludentes). Regarding the contribution of astrocytes to the BBB, it is well known that its end-feet form a relatively complete layer, but the junctions between are gap junctions and not of the occluding kind [35]. Perivascular end-feet of astrocytes do not provide an effective barrier even if substances should pass through the endothelial cells into the brain. Indeed, after intravenous infusion of peroxidase, endothelial cells show micropinocytotic vesicles containing the tracer [36]. Such a transendothelial cell barrier is very selective and based on carrier-mediated transports, but is not furtherly mechanically regulated.

Caveolae are small, bulb-shaped, plasma membrane invaginations. They have been described to have a function in endocytosis and transcytosis and in maintaining the lipid composition of the membrane, as well as acting as signaling background.

While endothelial cells in peripheral organs, such as the lung and heart, are enriched in caveolae, in BBB only a small number of caveolae are detectable [24]. Mfsd2a (transporter of the major facilitator superfamily domain-containing (Mfsd) family) contributes to the regulation of vesicular traffic in BBB endothelial cells [37] through the transport of the essential omega-3 fatty acid docosahexaenoic acid (DHA). The expression of Mfsd2a becomes upregulated in ECs with the maturation of BBB. Gene ablation of Mfsd2a in mice results in BBB leakiness and increased vesicular traffic in ECs.

Caveolins (Cavs) are thought to play a role in the regulation of BBB function. Cavs, are a family of integral membrane proteins which represent both positive and negative regulators of intracellular signaling as scaffolding proteins that regulate the intracellular distribution of the signaling molecules. Cav-1 overexpression protects the integrity of the BBB mainly by preventing the degradation of TJ proteins in rats [38].

Cav-1 is a marker of caveolae in endothelial cells and is important in the regulation of various functions like endocytosis, transcytosis, signal transduction, and molecular transport. Recent studies on mice indicate that the suppression of the caveolae pathway requires the transport of lipids, notably DHA-containing phospholipids, by Mfsd2a to regulate CNS endothelial cell plasma membrane composition and to inhibit caveolae vesicle formation [39].

Moreover, Cav-1 regulate the angiogenic response by influencing VEGF receptor 2 (VEGFR2) phosphorylation and internalization [40, 41].

In BBB the basement membrane (BM) represents the noncellular component. Astrocytes, PCs and ECs synthesize and secrete molecules which constitute the BM surrounding the external surface of the endothelial cell, composed by type IV collagen, fibronectin, heparan sulfate,

nidogen, osteonectin and laminin. BM functions as a charge and molecular weight barrier and is able to interact with integrins in order to regulate permeability and cellular transport also across the BBB. Dystroglycans and integrins are transmembrane receptors that allow BBB cells to interact with BM. During brain angiogenesis in mice, ECs show $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrin, whereas in adult animals $\alpha 4$ and $\alpha 5\beta 1$ integrins promote stabilization of vessels [42], $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrin induce cell proliferation through MAPK signaling in human ECs. Moreover, $\beta 1$ integrin interaction with laminin maintains levels of claudin-5 in TJs [43, 44].

3. Blood-brain barrier damage

The endothelial cells (ECs) compose the wall of vessels and capillaries and represent the primary blood-tissue barrier. The ECs acting as a protective filter are able to regulate the passage of molecules and immune cells and the level of specialization of each blood-barrier is determined by the functions of endothelial-wall. In the brain, there is the higher level of specialization of the endothelial wall. The filter function of BBB is carried out by ECs strictly interconnect with numerous tight junctions.

Pathological conditions within the central nervous system like ischemia, inflammation or tumor growth lead to blood-brain barrier (BBB) dysfunction, emphasizing that the permeability barrier regulation is principally provided by the local microenvironment and its maintenance is a necessary condition in any circumstance. In many brain tumors morphological irregularities of the perivascular space correlate with a breakdown of the BBB [45–47].

Wolburg et al. [48] found that claudin-3, a key component of BBB tight junctions, is lost in glioblastoma. Further evidence on claudin-1 loss in tumor microvessels, as well as downregulation of claudin-5 and occludin in hyperplastic vasculature, result in a phenotypic change in BBB function due to leaky tight junctions and hyperpermeable endothelial cells.

A recent study by Watkins et al. [49] using a mouse model demonstrates that astrocytic end-feet displace from their position alongside endothelial cells with disruption of the communication between the astrocytes and vasculature and that single glioma cells were sufficient to produce local BBB opening.

The structural perturbations of the vascular barrier during tumor progression, in other organs was demonstrated, are successive to the release in the tumor microenvironment of specific cytokines that downregulate the transcription of these structural proteins [50].

The crucial point is the vascular damage that the tumor direct and indirect operates through the release on the next microenvironment of chemical factors able to increase the permeability of the tissue vessels reducing their protective barrier function. This could explain the pathogenesis of the BBB damage occurring during the development of glioblastoma. The BBB damage induced by the glioblastoma represents a strategy employed to control the BBB opening to allow the passage of drugs [51].

The detection of endogenous circulating molecules normally restricted by the BBB, namely albumin, immunoglobulin G, or fibrinogen, in the brain parenchyma, using immunohistochemistry

or immunofluorescence is one of the most straightforward in situ techniques used to assess BBB impairment [52].

Measurement of exogenous tracer extravasation remains a technique of choice in preclinical studies testing for BBB permeability. A variety of detectable exogenous tracers has become available, and methods have been developed to evaluate the kinetics of their extravasation. Due to variations in tightness of the BBB, the extravasation of different BBB permeability tracers across an impaired BBB critically depends on their physicochemical properties and molecular weight.

In recent years, different in vivo imaging techniques have contributed to the understanding of BBB dysfunction in disease [52]. Among these, noteworthy:

- In vivo confocal microscopy, an important tool for the high-resolution dynamic fluorescence imaging in BBB research. This technique has the advantage of time-lapse imaging of the same subject. The apparent disadvantages of this technique are the technical complexity and invasive nature, because it requires opening of a cranial window in experimental animals.
- Noninvasive fluorescence imaging (NFI), image acquisition is fast (in the range of seconds), and the imaging equipment is comparably inexpensive, simple, and easy to use. However, NFI has a limited resolution (1–2 mm) and quantitation of the imaging signal has several pitfalls.
- Nuclear imaging, rarely used for clinical imaging of BBB impairment. Examples of nuclear imaging techniques are single photon emission computed tomography (SPECT) and positron emission tomography (PET).
- Magnetic resonance imaging, MRI upon intravenous injection of a contrast agent, usually gadolinium-diethylene triamine pentaacetic acid (Gd-DTPA; MW 550 Da), is the most commonly used noninvasive imaging technique for detection of BBB impairment in both clinical and preclinical studies.

With the exception of brain tumors, where angiogenic biomarkers have been targeted for molecular imaging [53], cerebrovascular biomarkers have not yet been fully exploited for molecular imaging of brain diseases.

Despite this advance the problem to track BBB opening remains for insufficient selectivity, specificity of the tools provided and for the no sufficiently abundance [52] of the biologics-based molecular imaging probes today available. In this prospective the liquid biopsy could represent an opportunity to track in real time the BBB opening. The concept is based on the causal release of tumor cells in the systemic circulation through the BBB damage. The structural perturbation of the BBB and the consequent modification of its permeability could induce cell spread independently by the molecular equipment expressed by cancer cell. In this direction, the detection of the circulating cancer cells in glioblastoma cases could acquire a specific significance related to the permeability of the BBB more than a prognostic value related to the dissemination phase of the disease (**Figure 2**).

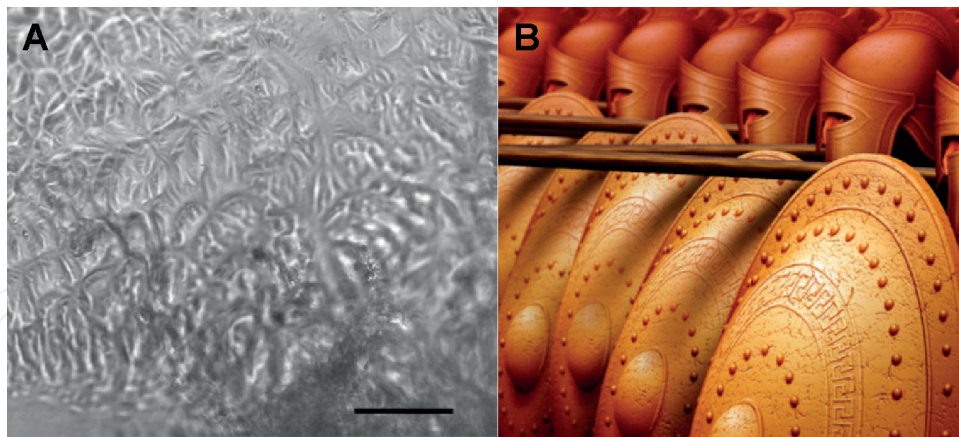


Figure 2. Vessel's arrangement of endothelial cells acting as protective filter (A) mimics an array of warriors (B) placed to protect the tissues. In (A), primary human endothelial culture. Scale bar 100 μm (figure partially modified from Malara et al. *IJC Heart & Vasculture* [106]).

4. Glioblastoma's angiogenesis

Gliomas are a heterogeneous group of neoplasms derived from glial cells that account for 40–45% of all intracranial tumors. The most malignant type of glioma, Glioblastoma multiforme (GBM), account for approximately 12–15% of all intracranial neoplasms and 50–60% of all astrocytic tumors. In most European and North American countries, incidence is approximately 2–3 new cases per 100,000 people per year. Nearly 260,000 patients worldwide are diagnosed annually with primary malignant brain cancer [54, 55]. The World Health Organization defines GBM as a cancer of grade IV characterized as malignant, mitotically active, and predisposed to cellular necrosis [56].

Untreated patients with glioblastoma multiforme uniformly die within 3 months from the diagnosis. Treated patients with a protocol including surgical resection, radiation therapy, and chemotherapy, have a median survival of 14.6 months, with fewer than 25% of patients surviving up to 2 years and fewer than 10% up to 5 years [52, 57, 58]. Moreover, males had a slight preponderance over females, with a male-to-female ratio of 1.6:1. GBM may manifest in persons of any age, but it affects adults preferentially, with a peak incidence at 45–70 years [55]. Glioblastomas are tumors that display extensive morphological and molecular heterogeneity, and thus may reflect their origin from different population of astrocytes, and possibly from oligodendrocytes and ependymal cell lineages. GBM rarely metastasize outside the brain [59]. GBM can be classified into primary type and secondary type, according to whether they are generated *de novo* or by progression of lower-grade tumors. Histologically, primary and secondary glioblastomas are largely indistinguishable, but they differ in their genetic and epigenetic profiles [60]. GBM is a highly aggressive tumor with distinct histopathological features, including high proliferation, necrosis and considerable neovascularization (i.e., angiogenesis), leading to vessels that exhibit morphological abnormalities and “leakiness” [60]. It is generally accepted that the degree of angiogenesis is correlated to the malignancy of the tumor [58, 61]. GBMs are the most lethal cancer and the most vascularized brain cancer, with

the highest degree of vascular proliferation and endothelial cell hyperplasia [62]. Patients with high tumor microvascular densities exhibit shorter postoperative survival rates than patients with low microvascular densities [63, 64].

Angiogenesis and tumor cell invasion play a critical role in GBM development and growth, even during the earliest phases [65, 66].

Angiogenesis requires three distinct steps: (1) blood vessel breakdown, (2) degradation of the vessel basement membrane and the surrounding extracellular matrix (ECM), and (3) migration of endothelial cells for the formation of new blood vessels. The first step (1) in forming new blood vessels from existing vessels is the dissolution of aspects of native vessels [67].

Tumor angiogenesis results from a balance between pro-angiogenic factors and anti-angiogenic factors with a shift toward angiogenic factors that stimulate uncontrolled and disorganized vascular growth. These molecular factors can be secreted by cancer, endothelial, stromal, and blood cells and by the extracellular matrix [68].

Pro-angiogenic factors include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), acidic fibroblast growth factor, basic fibroblast growth factor (bFGF), placental growth factor, angiopoietin-2 (Ang-2), and interleukins, whereas anti-angiogenic factors include angiostatin, endostatin, thrombospondin 1, and endothelial monocyte-activating polypeptide 2 [69, 70].

The VEGF play a major role in GBM angiogenesis by stimulating capillary sprouting from pre-existing vessels toward VEGF-expressing tumor cells. Tumor VEGF expression and angiogenesis are mainly hypoxia-driven, but can also be promoted by other vascular cytokines and constitutively expressed by genetic tumor mutations [71].

Another mechanism of neo-vascularization includes the recruitment of endothelial progenitor cells (EPCs) that have been proposed to originate from different sources, including bone marrow, the existing vasculature, or adipose tissue [72, 73].

Upon BBB injury, endothelial progenitor cells (EPC) contribute (directly or as a source and carrier of pro-angiogenic factors) to BBB re-endothelialization. EPC are involved to the cerebral angiogenesis associated with physiological conditions (i.e., activity-induced neurogenesis) or pathological conditions (i.e., tumor progression). In adults, there are three origins of EPC:

- i. Bone marrow origin (EPC derived from bone marrow multipotent hemangioblasts [VEGFR2(+)/VEcadherin(+)/CD45(-)]
- ii. Mesenchymal origin SC) (CD73(+)/CD90(+)/CD105(+)/CD34(-)/CD45(-)];
- iii. Tissue origin (EPC found at the sites of extensive angiogenesis but demonstrating no signs of hematopoietic origin, being, probably, derived from tissue multipotent cells) [73–75]

In pathological conditions, angiogenesis and vascular remodeling are usually considered as significant components of brain tissue repair program after injury (hypoxic, ischemic, traumatic, inflammatory, toxic, etc.) and the mobilization of EPC from bone marrow correlates to the severity of cerebral alterations [76].

A key challenge in the field of BBB permeability is the current shortage of the knowledge on the most efficient way to cross BBB used by tumor cells. It has been suggested that the brain endothelial cells can actively participate in metastatic progression and stimulate increased BBB permeability.

During vasculogenesis, EPCs are mobilized from the bone marrow by increased concentrations of chemokines, growth factors and other soluble factors in serum, including stromal cell-derived factor-1 (SDF-1), VEGF, and granulocyte-monocyte colony stimulating factor (GM-CSF) [77, 78].

Given the high rate of angiogenesis in glioblastomas and the lack of prognostic markers for anti-VEGF treatments in general, the kinetics and prognostic relevance of circulating endothelial cells was assessed relatively to their potential role. It is clear that molecules with high pro-angiogenic potential provide the recruitment of EPC from bone marrow (VEGF, IL-8, IGF, etc.) [81]. Moreover, EPC provide paracrine signaling to facilitate angiogenesis [79, 80]. It has been suggested that quantification of CECs is useful to identify patients who might benefit from anti-angiogenic treatments [81]. Batchelor et al., in a series of patients with glioblastoma treated with AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, found that viable CEC number increased when tumors escaped treatment [82]. Indeed, CECs, rare in healthy individuals, increase in vascular disorders and tumors due to vascular damage such as the GBM, in which, the VEGF is highly expressed and may mobilize endothelial precursors from the bone marrow [83–85]. During GBM progression, the extracellular matrix (ECM) changes its flexibility and structure (i.e., content of fibrillary proteins as collagen, fibronectin, etc.) and malignant infiltration of glioma may stimulate the development of distinct provisional ECM patterns. Indeed, tumor cells induce changes in ECM by secretion of growth factors and angiogenic factors such as VEGF, bFGF, PDGF, and TNF- α [86]. The combined release of these factors have a synergic effect on the mobilization of EPC [87]. Further studies are needed to assess the role of EPC in liquid biopsy as marker of BBB dysfunction.

5. Microenvironment in glioblastoma

Cytokines play a critical role in contributing to the complexity and lethality of glioblastoma characterized by diffuse invasiveness, immunosuppression, aggressive proliferation, vascularization, and resistance to conventional radiotherapy and chemotherapy. Cytokines consist in small molecules with diverse effects depending on the microenvironment. Cytokines comprise glycoproteins and polypeptides that exert pro-inflammatory, anti-inflammatory or immunosuppressive action. In addition to the tumor cells, immune cells, extracellular matrix, blood vessels, the cytokines are an integral part of the network GBM tumor microenvironment-associated. Glioblastoma arises from astrocytes and their precursors, neural stem cells. The resulting tumor is a heterogeneous cellular population composed of both undifferentiated and differentiated cells and containing subpopulations of tumor cell with different grade of tumorigenic property. Recently, study on GBM progression reveals a feed-forward mechanism with the epidermal growth factor receptor variant III (EGFRvIII) and STAT3 defined by the presence of cytokine receptor OSMR on the surface of non-tumorigenic and tumorigenic brain tumor cells.

In particular, EGFRvIII-OSMR complex signals activates STAT3, and STAT3 signals upregulates OSMR expression. The result is a feed-forward signaling mechanism that drives oncogenesis in GBM. One of principal activator of the JAK-STAT signaling pathway is IL-6. Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates the immune response, but also plays a role in promoting tumor growth and survival [88, 89]. In gliomas, the level of IL-6 gene expression increases with the grade of malignancy. In GBM, the, amplification/overexpression of the IL-6 gene appears to be a common feature [90]. Array studies have reported that the number of IL-6 gene copies was increased in 40–50% of GBM [91, 92]. In ependymoma, with inflammatory phenotype, was described a constitutive activation of the IL6/STAT3 pathway and crosstalk between tumor and immune cells with significant increase in STAT3 and IL8 secretion in tumor microenvironment. Moreover, IL-6 and IL-8, EGF, and other cytokines are involved on the regulation of endothelial function at BBB level, with several potential outcomes: increased permeability, generation of relaying signaling including another cytokine(s) and soluble mediators potentiating the effects of another cytokine(s), modulation of efflux transporters). Even though the BBB within the tumor is considered “permeable,” in large parts of gliomas the BBB more closely resembles the intact BBB and prevents efficient passage of cancer therapeutics, such as small molecules and antibodies. Recently, was reported a tumor screening study that combined the detection and analysis of circulating tumor DNA with protein markers comprising some interleukins and the increase of chemokines (IL-6, IL-8) with encouraging results [93]. The big limit in the use of circulating tumor DNA in the screening phase of tumor disease is due to the poor specificity of this biomarker. In fact, mutated circulating DNA may be found in the peripheral blood of healthy subjects, it represents the genetic segments discarded during the normal proliferation of the cells in the body, for example during the hematopoiesis.

6. Circulating biomarkers of glioblastoma

The dissemination of cancer cells in the bloodstream transforms a limited disease in a systemic pathology with remarkable prognostic and therapeutic complications for the patients. The phase of dissemination is a complex plan of action, in which, independently by type of tumor and its location, there are two main actors, the endothelial and the tumor cells. The endothelial cells compose the wall of vessels and capillaries and represent the primary blood-tissue barrier. The adhesion molecules expressed on ECs surface condition the permeability and trans-endothelial resistance to cell crossing [6].

In the brain, the filter function of BBB is carried out by ECs strictly interconnect with numerous tight junctions. The tight junctions give continuity to the endothelial wall eliminating the intercellular space. Astrocyte end-feet, pericytes and microglia interacting with ECs draining fluids in the glymphatic system. Glymphatic system controls the levels of concentration of solutes in the neuronal interstitium and it is connected with the blood circulation [6].

Animal models combined with observations on humans, are still promising tools in order to clarify mechanisms of passage of malignant cells through the BBB. Indeed, similarly to what is observed in epithelial tumors have been demonstrated by patient-derived GBM xenografts that acquisition of a mesenchymal phenotype is mandatory for malignant cells in order to enter the blood stream and behavior as CTCs [94].

The detection of CTCs in the field of CNS represents a promising noninvasive technique to facilitate early diagnosis and monitoring tumor evolution [95, 96]. The significance of the CTCs detection in intracranial tumors, commonly related to metastatic phase of the cancer disease, was hardly applied for the very few case with extracranial metastases observed although the high malignancy and invasiveness of glioma [97]. More recently, it was reported that disseminating cells of medulloblastoma were able to infiltrate the leptomeninges through hematological way [98]. Garzia et al. described an in vivo experiment consisting in *via surgical* union of two mice by their flanks. The cancer cells of medulloblastoma implanted in one mouse model were able via hematological to give leptomeningeal metastatic disease in the other one mouse. The surgical union shared not only the cells but also the biochemical environment. The biochemical composition of cancer microenvironment consists by a mixture of permeabilizing and inflammatory cytokines. Many types of cytokines like Histamine, TNF α , IL1 β and in particular the axis, chemokine CCL2-CCR2 receptor, sustain BBB alterations. Their action plays on BBB by activating cytoskeleton redistribution, downregulating structural proteins and favoring the tight-junction opening [99]. Garzia et al. to obtain leptomeningeal relapsing lesion used an experiment that shared the microenvironment rather than to inject only cancer cells. The tumor cells, although expressing molecular equipment involved in cell movement, were not able, alone, to determine leptomeningeal metastasis. The tumor cytokines mediated BBB-opening and successively favored cellular BBB-crossing.

Moreover, due to the contribution of other studies demonstrating that CTCs can be detected in all pathological subtypes of glioma, irrespective of different malignant degree, now, the reductive misconception of *metastatic* CTCs has been challenged suggesting that CTCs should be considered a common property of glioma lesions [100]. The behavior of the CTCs suggest that the pathogenesis of their release in the blood stream is independent from the grade of malignancy of intracranial tumor, and such as happened in other tumor diseases, this phenomenon happened in each phase of cancer development [101].

The BBB confers to intracranial tumor diseases a different nosology. The presence of CTCs in the bloodstream of patients with intracranial tumors acquires a diverse significance respect to cancers located in extra-cranial sites. Regarding the intracranial cancers could be more appropriate to use CTCs as biomarkers of BBB-damage rather than biomarkers of a preferential/alternative dissemination way. The understanding of this scenario is important to focus the better targeting strategy. The interference on the interaction between cancer-endothelial cells or the reduction of the cytokines levels could be the key targets for pharmacological interventions for the inhibition of cell spread by an intracranial tumor.

Another emerging role in the study of glioblastoma and liquid biopsy are represented by the detection in the peripheral blood of extracellular vesicles (EVs) of tumor origin. EVs are secreted by tumor cells, are mediators of intercellular communication that transfer nucleic acids, proteins and lipids. The liquid biopsy of EVs in patients with GBM has the role to clarify their implications in tumor progression, as a tumor biomarker for tracking GBM progression and as a potential therapeutic target/delivery system [102]. Therapeutic experimental protocols involving EVs delivery of miRNA were performed for GBM. The deletions on chromosome 10 are a common chromosomal alteration found within GBM with leads to a loss of miR-146b normally located at 10q24 [103]. In GBM the loss of miR-146b facilitates migration and invasion. Moreover, in a study using an in vivo model, the exosomes derived



Figure 3. Schematic representations of endothelial-cancer cells interaction. (A) Interstitium space at brain (blood-brain barrier, BBB) and at blood barrier (BB) of other organs. Vessels (red), pericyte (green-cell), astrocyte end-feet (yellow-cell) and cancer cells (blue cells). (B) Cancer cells cross BB through pre-existing inter-endothelial spaces, (C) while at BBB overcome the absence of intercellular spaces realizing permeabilizing cytokines (green balls).

from marrow cells were administered in intracranial rat tumors with consequent reduction of glioma cell invasion, migration, viability and expression of EGFR normally amplified in approximately 40% of all GBM [104]. The possibility of miRNA-based approaches using exosomes have a strong therapeutic potential, which could be achieved through the delivery and transfer agents in those cases of GBM in which the BBB opening is a rare event. In these cases, the liquid biopsy negative for the detection of EPC and CTCs could be useful to select the patients eligible to this type of approach (**Figure 3**).

7. Conclusive remarks

In conclusion, the answers to the question when and why the liquid biopsy is useful in glioblastoma disease, could be different in function of the phase of disease:

1. Diagnostic glioblastoma phase: the liquid biopsy, searching and characterizing the circulating glial cells, could discriminate between glioblastoma and other intracranial lesions, supporting the clinical imaging investigations and in substitution of the stereotactic biopsy procedure when this procedure is at high risk of side effects.
2. Prognostic evaluation and monitoring phase: the prognostic value of the CTCs in glioblastoma patients should be clarified by further studies. Their release in the bloodstream not should be considered as a marker of dissemination and consequently of a bad prognosis. Their detection in the blood is in function of the particular anatomic location of cancer inducing local structural perturbations and only partially dependent by its grade of malignancy. In fact, the release of CTCs in the blood is conditioned by many local factors such as peritumor inflammation reaction, structural changes on the BBB, altered cancer microenvironment, etc. On this way, could be better to analyze the circulating segments of tumor DNA, carrying of hotspot mutations specifically related to glioblastoma, to define prognosis and to monitor during the treatment the emergence of aberrant mutations in advance to predict chemo resistance.
3. Anticancer-treatment design phase: the combining detection of CTCs and EPCs as markers of the opening status of BBB could be useful to individuate the better timing of treatment,

in which therapeutic drugs may be favorite to cross BBB. Moreover, those patients in which the BBB opening is not detectable through liquid biopsy could be considered a different therapeutic approach using EVs.

Author details

Natalia Malara* and Giuseppe Donato

*Address all correspondence to: nataliamalara@unicz.it

School of Medicine, University Magna Graecia of Catanzaro, Italy

References

- [1] Lampson LA, Lampson MA, Dunne AD. Exploiting the lacZ reporter gene for quantitative analysis of disseminated tumor growth within the brain: Use of the lacZ gene product as a tumor antigen, for evaluation of antigenic modulation, and to facilitate image analysis of tumor growth in situ. *Cancer Research*. 1993;**53**(1):176-182
- [2] Lund CV, Nguyen MT, Owens GC, Pakchoian AJ, Shaterian A, Kruse CA, et al. Reduced glioma infiltration in Src-deficient mice. *Journal of Neuro-Oncology*. 2006;**78**(1):19-29
- [3] Winkler F, Kienast Y, Fuhrmann M, Von Baumgarten L, Burgold S, Mitteregger G, et al. Imaging glioma cell invasion in vivo reveals mechanisms of dissemination and peritumoral angiogenesis. *Glia*. 2009;**57**(12):1306-1315. DOI: 10.1002/glia.20850
- [4] Rehfeld A, Nylander M, Karnov K. *Compendium of Histology*. Switzerland: Springer; 2017. pp. 298-299
- [5] Engelhardt B, Vajkoczy P, Weller RO. The movers and shapers in immune privilege of the CNS. *Nature Immunology*. 2017;**18**(2):123-131. DOI: 10.1038/ni.3666
- [6] Louveau A, Plog BA, Antila S, Alitalo K, Nedergaard M, Kipnis J. Understanding the functions and relationships of the glymphatic system and meningeal lymphatics. *The Journal of Clinical Investigation*. 2017;**127**(9):3210-3219. DOI: 10.1172/JCI90603
- [7] Obermeier B, Daneman R, Ransohoff RM. Development, maintenance and disruption of the blood-brain barrier. *Nature Medicine*. 2013;**19**(12):1584-1596. DOI: 10.1038/nm.3407
- [8] Flamme I, Frölich T, Risau W. Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *Journal of Cellular Physiology*. 1997;**173**(2):206-210
- [9] Raab S, Beck H, Gaumann A, Yüce A, Gerber HP, Plate K, et al. Impaired brain angiogenesis and neuronal apoptosis induced by conditional homozygous inactivation of vascular endothelial growth factor. *Thrombosis and Haemostasis*. 2004;**91**(3):595-605

- [10] Tata M, Ruhrberg C, Fantin A. Vascularisation of the central nervous system. *Mechanisms of Development*. 2015;**138**(Pt 1):26-36
- [11] Haigh JJ, Morelli PI, Gerhardt H, Haigh K, Tsien J, Damert A, et al. Cortical and retinal defects caused by dosage-dependent reductions in VEGF-A paracrine signaling. *Developmental Biology*. 2003;**262**(2):225-241
- [12] Geudens I, Gerhardt H. Coordinating cell behaviour during blood vessel formation. *Development*. 2011;**138**(21):4569-4583. DOI: 10.1242/dev.062323
- [13] Hellström M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*. 2007;**445**(7129):776-780
- [14] Goodwin AM, D'Amore PA. Wnt signaling in the vasculature. *Angiogenesis*. 2002;**5**:1-9
- [15] Muley A, Majumder S, Kolluru GK, Parkinson S, Viola H, Hool L, et al. Secreted frizzled-related protein 4: An angiogenesis inhibitor. *The American Journal of Pathology*. 2010;**176**:1505-1516. DOI: 10.2353/ajpath.2010.090465
- [16] Miller JR. The Wnts. *Genome Biology*. 2002;**3**(1) REVIEWS3001
- [17] Cadigan KM. Wnt/beta-catenin signaling: Turning the switch. *Developmental Cell*. 2008;**14**(3):322-323. DOI: 10.1016/j.devcel.2008.02.006
- [18] Olsen JJ, Pohl SÖ, Deshmukh A, Visweswaran M, Ward NC, Arfuso F, et al. The role of Wnt signalling in angiogenesis. *Clinical Biochemist Reviews*. 2017;**38**(3):131-142
- [19] Schulzke JD, Günzel D, John LJ, Fromm M. Perspectives on tight junction research. *Annals of the New York Academy of Sciences*. 2012;**1257**:1-19. DOI: 10.1111/j.1749-6632.2012.06485.x
- [20] Feldman GJ, Mullin JM, Ryan MP. Occludin: Structure, function and regulation. *Advanced Drug Delivery Reviews*. 2005;**25**(57(6)):883-917
- [21] Engelhardt B, Liebner S. Novel insights into the development and maintenance of the blood-brain barrier. *Cell and Tissue Research*. 2014;**355**(3):687-699. DOI: 10.1007/s00441-014-1811-2
- [22] Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *The Journal of Cell Biology*. 2003;**161**:653-660
- [23] Caley DW, Maxwell DS. Development of the blood vessels and extracellular spaces during postnatal maturation of rat cerebral cortex. *The Journal of Comparative Neurology*. 1970;**138**:31-47
- [24] Iwamoto N, Higashi T, Furuse M. Localization of angulin-1/LSR and tricellulin at tricellular contacts of brain and retinal endothelial cells in vivo. *Cell Structure and Function*. 2014;**39**(1):1-8
- [25] Fiorentino M, Sapone A, Senger S, Camhi SS, Kadzielski SM, Buie TM, et al. Blood-brain barrier and intestinal epithelial barrier alterations in autism spectrum disorders. *Molecular Autism*. 2016;**7**:49

- [26] Sohet F, Lin C, Munji RN, Lee SY, Ruderisch N, Soung A, et al. LSR/angulin-1 is a tricellular tight junction protein involved in blood-brain barrier formation. *The Journal of Cell Biology*. 2015;**208**(6):703-711. DOI: 10.1083/jcb.201410131
- [27] Munde PB, Khandekar SP, Dive AM, Upadhyaya NR. Pericytes in health and disease. *International Journal of Oral & Maxillofacial Pathology*. 2014;**5**(1):02-07
- [28] Liebner S, Dijkhuizen RM, Reiss Y, Plate KH, Agalliu D, Constantin G. Functional morphology of the blood-brain barrier in health and disease. *Acta Neuropathologica*. 2018;**135**(3):311-336. DOI: 10.1007/s00401-018-1815-1
- [29] Sweeney MD, Ayyadurai S, Zlokovic BV. Pericytes of the neurovascular unit: Key functions and signaling pathways. *Nature Neuroscience*. 2016;**19**(6):771-783. DOI: 10.1038/nn.4288
- [30] Chen J, Luo Y, Hui H, Cai T, Huang H, Yang F, et al. CD146 coordinates brain endothelial cell-pericyte communication for blood-brain barrier development. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;**114**(36):E7622-E7631. DOI: 10.1073/pnas.1710848114
- [31] Yao Y, Chen ZL, Norris EH, Strickland S. Astrocytic laminin regulates pericyte differentiation and maintains blood brain barrier integrity. *Nature Communications*. 2014;**5**:3413. DOI: 10.1038/ncomms4413
- [32] Cheslow L, Alvarez JI. Glial-endothelial crosstalk regulates blood-brain barrier function. *Current Opinion in Pharmacology*. 2016;**26**:39-46. DOI: 10.1016/j.coph.2015.09.010
- [33] Dempsey EW, Wislocki GB. An electron microscopic study of the blood-brain barrier in the rat, employing silver nitrate as a vital stain. *The Journal of Biophysical and Biochemical Cytology*. 1955;**1**(3):245-256
- [34] Leonhardt H. On the capillaries and the perivascular structures of the area postrema of the rabbit and on its behavior in pentamethylenetetrazol- (cardiazol-) convulsion. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*. 1967;**76**(4):511-524
- [35] Peters A, Palay SL, Webster H. *The Fine Structure of the Nervous System*. Oxford: Oxford University Press; 1991. pp. 291-293
- [36] Reese TS, Karnovsky MJ. Fine structural localization of a blood-brain barrier to exogenous peroxidase. *The Journal of Cell Biology*. 1967;**34**(1):207-217
- [37] Ben-Zvi A, Lacoste B, Kur E, Andreone BJ, Mayshar Y, Yan H, et al. Mfsd2a is critical for the formation and function of the blood-brain barrier. *Nature*. 2014;**509**(7501):507-511. DOI: 10.1038/nature13324
- [38] Choi KH, Kim HS, Park MS, Lee EB, Lee JK, Kim JT, et al. Overexpression of caveolin-1 attenuates brain edema by inhibiting tight junction degradation. *Oncotarget*. 2016;**7**(42):67857-67867. DOI: 10.18632/oncotarget.12346
- [39] Andreone BJ, Chow BW, Tata A, Lacoste B, Ben-Zvi A, Bullock K, et al. Blood-brain barrier permeability is regulated by lipid transport-dependent suppression of

- Caveolae-mediated Transcytosis. *Neuron*. 2017;**94**(3):581-594.e5. DOI: 10.1016/j.neuron.2017.03.043
- [40] Zenaro E, Piacentino G, Constantin G. The blood-brain barrier in Alzheimer's disease. *Neurobiology of Disease*. 2017;**107**:41-56. DOI: 10.1016/j.nbd.2016.07.007
- [41] Carvey PM, Hendey B, Monahan AJ. The blood-brain barrier in neurodegenerative disease: A rhetorical perspective. *Journal of Neurochemistry*. 2009;**111**(2):291-314. DOI: 10.1111/j.1471-4159.2009.06319.x
- [42] Wang J, Milner R. Fibronectin promotes brain capillary endothelial cell survival and proliferation through $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins via MAP kinase signalling. *Journal of Neurochemistry*. 2006;**96**(1):148-159
- [43] Milner R, Campbell IL. Developmental regulation of $\beta 1$ integrins during angiogenesis in the central nervous system. *Molecular and Cellular Neurosciences*. 2002;**20**(4):616-626
- [44] Osada T, Gu YH, Kanazawa M, Tsubota Y, Hawkins BT, Spatz M, et al. Interendothelial claudin-5 expression depends on cerebral endothelial cell-matrix adhesion by $\beta (1)$ -integrins. *Journal of Cerebral Blood Flow and Metabolism*. 2011;**31**(10):1972-1985. DOI: 10.1038/jcbfm.2011.99
- [45] Millauer B, Witzigmann-Voos S, Plate H, Risau W, Ullrich A. Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. *Nature*. 1994;**367**(6463):576-579
- [46] Plate KH, Breier G, Weich A, Risau W. Vascular endothelial growth-factor is a potential tumor angiogenesis factor in human gliomas in vivo. *Nature*. 1992;**359**(6398):845-848
- [47] Risau W. Development of blood-brain barrier endothelial cells. In: Couraud PO, Scherman D, editors. *Biology and Physiology of the Blood-Brain Barrier*. Boston: Springer; 1996. DOI: 10.1007/978-1-4757-9489-2_1
- [48] Wolburg H, Wolburg-Buchholz K, Kraus J, Rascher-Eggstein G, Liebner S, Hamm S, et al. Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme. *Acta Neuropathologica*. 2003;**105**:586-592
- [49] Watkins S, Robel S, Kimbrough IF, Robert SM, Ellis-Davies G, Sontheimer H. Disruption of astrocyte-vascular coupling and the blood-brain barrier by invading glioma cells. *Nature Communications*. 2014;**5**:4196. DOI: 10.1038/ncomms5196
- [50] Suzuki T, Yoshinaga N, Tanabe S. Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *The Journal of Biological Chemistry*. 2011;**286**(36):31263-31271. DOI: 10.1074/jbc.M111.238147
- [51] Jain KK. Nanobiotechnology-based strategies for crossing the blood-brain barrier. *Nanomedicine*. 2012;**7**(8):1225-1233. DOI: 10.2217/nnm.12.86
- [52] Wunder A, Schoknecht K, Stanimirovic DB, Prager O, Chassidim Y. Imaging blood-brain barrier dysfunction in animal disease models. *Epilepsia*. 2012;**53**(6):14-21. DOI: 10.1111/j.1528-1167.2012.03698.x

- [53] Cai W, Chen K, Mohamedali KA, Cao Q, Gambhir SS, Rosenblum MG, et al. PET of vascular endothelial growth factor receptor expression. *Journal of Nuclear Medicine*. 2006;**47**(12):2048-2056
- [54] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer*. 2015;**136**:E359-E386. DOI: 10.1002/ijc.29210
- [55] Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial, *Lancet Oncology*. 2009;**10**(5):459-466. DOI: 10.1016/S1470-2045(09)70025-7
- [56] McLendon RE, Halperin EC. Is the long-term survival of patients with intracranial glioblastoma multiforme overstated? *Cancer*. 2003;**98**(8):1745-1748
- [57] Ricard D, Idbaih A, Ducray F, Lahutte M, Hoang-Xuan K, Delattre JY. Primary brain tumours in adults. *Lancet*. 2012;**379**(9830):1984-1996. DOI: 10.1016/S0140-6736(11)61346-9
- [58] Grossman SA, Norris LK. Adjuvant and neoadjuvant treatment for primary brain tumors in adults. *Seminars in Oncology*. 1995;**22**:530-539
- [59] Hayat MA. Introduction. In: Hayat MA, editor. *Tumors of the Central Nervous System*. Berlin: Springer; 2014. pp. 3-4
- [60] Vajkoczy P, Menger MD. Vascular microenvironment in gliomas. *Journal of Neuro-Oncology*. 2000;**50**(1-2):99-108
- [61] Daumas-Duport C, Tucker ML, Kolles H, Cervera P, Beuvon F, Varlet P, et al. Oligodendrogliomas. Part II: A new grading system based on morphological and imaging criteria. *Journal of Neuro-Oncology*. 1997;**34**:61-78
- [62] Brem S, Cotran R, Folkman J. Tumor angiogenesis: A quantitative method for histologic grading. *Journal of the National Cancer Institute*. 1972;**48**(2):347-356
- [63] Birlik B, Canda S, Ozer E. Tumour vascularity is of prognostic significance in adult, but not paediatric astrocytomas. *Neuropathology and Applied Neurobiology*. 2006;**32**(5):532-538
- [64] Leon SP, Folkerth RD, Black PML. Microvessel density is a prognostic indicator for patients with astroglial brain tumors. *Cancer*. 1996;**77**(2):362-372
- [65] Bello L, Giussani C, Carrabba G, Pluderi M, Costa F, Bikfalvi A. Angiogenesis and invasion in gliomas. *Cancer Treatment and Research*. 2004;**117**:263-284
- [66] Cea V, Sala C, Verpelli C. Antiangiogenic therapy for glioma. *Journal of Signal Transduction*. 2012;**2012**:483040. DOI: 10.1155/2012/483040
- [67] Zagzag D1, Amirnovin R, Greco MA, Yee H, Holash J, Wiegand SJ, et al. Vascular apoptosis and involution in gliomas precede neovascularization: A novel concept for glioma growth and angiogenesis. *Laboratory Investigation*. 2000;**80**(6):837-849

- [68] Tandle A, Blazer DG 3rd, Libutti SK. Antiangiogenic gene therapy of cancer: Recent developments. *Journal of Translational Medicine*. 2004;**2**(1):22
- [69] Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science*. 1994;**265**(5178):1582-1584
- [70] Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, et al. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proceedings of the National Academy of Sciences of the United States of America*. 1990;**87**(17):6624-6628
- [71] Tuettenberg J, Friedel C, Vajkoezy P. Angiogenesis in malignant glioma—A target for antitumor therapy? *Critical Reviews in Oncology/Hematology*. 2006;**59**(3):181-193
- [72] Dome B, Dobos J, Tovari J, Paku S, Kovacs G, Ostoros G, et al. Circulating bone marrow-derived endothelial progenitor cells: Characterization, mobilization, and therapeutic considerations in malignant disease. *Cytometry. Part A*. 2008;**73**(3):186-193
- [73] Chao H, Hirschi KK. Hemato-vascular origins of endothelial progenitor cells? *Microvascular Research*. 2010;**79**(3):169-173. DOI: 10.1016/j.mvr.2010.02.003
- [74] Leeper NJ, Hunter AL, Cooke JP. Stem cell therapy for vascular regeneration: Adult, embryonic, and induced pluripotent stem cells. *Circulation*. 2010;**122**(5):517-526. DOI: 10.1161/CIRCULATIONAHA.109.881441
- [75] Boxall SA, Jones E. Markers for characterization of bone marrow multipotential stromal cells. *Stem Cells International*. 2012;**2012**:975871. DOI: 10.1155/2012/975871
- [76] Arai K, Jin G, Navaratna D, Lo EH. Brain angiogenesis in developmental and pathological processes: Neurovascular injury and angiogenic recovery after stroke. *The FEBS Journal*. 2009;**276**(17):4644-4652. DOI: 10.1111/j.1742-4658.2009.07176.x
- [77] Aicher A, Zeiher AM, Dimmeler S. Mobilizing endothelial progenitor cells. *Hypertension*. 2005;**45**(3):321-325
- [78] Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: Towards marker and target identification. *Nature Reviews. Cancer*. 2006;**6**(11):835-845
- [79] Caiado F, Carvalho T, Silva F, Castro C, Clode N, Dye JF, et al. The role of fibrin E on the modulation of endothelial progenitors adhesion, differentiation and angiogenic growth factor production and the promotion of wound healing. *Biomaterials*. 2011;**32**(29):7096-7105. DOI: 10.1016/j.biomaterials.2011.06.022
- [80] Zhang F, Xu CL, Liu CM. Drug delivery strategies to enhance the permeability of the blood–brain barrier for treatment of glioma. *Drug Design, Development and Therapy*. 2015;**9**:2089-2100. DOI: 10.2147/DDDT.S79592
- [81] Mancuso P, Calleri A, Bertolini F. Circulating endothelial cells and circulating endothelial progenitors. *Recent Results in Cancer Research*. 2012;**195**:163-170. DOI: 10.1007/978-3-642-28160-0_14

- [82] Batchelor TT1, Sorensen AG, di Tomaso E, Zhang WT, Duda DG, Cohen KS, et al. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell*. 2007;**11**(1):83-95
- [83] Plate KH, Breier G, Weich A, Risau W. Vascular endothelial growth-factor is a potential tumor angiogenesis factor in human gliomas in vivo. *Nature*. 1992;**359**:845-848
- [84] Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P, et al. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *The FASEB Journal*. 2006;**20**:1495-1497
- [85] Asahara T1, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *The EMBO Journal*. 1999;**18**(14):3964-3972
- [86] Gesa Rascher-Eggstein SL, Wolburg H. The blood-brain barrier in the human glioma. In: Shanka HS, Westman J, editors. *Blood-Spinal Cord and Brain Barriers in Health and Disease*. Amsterdam: Elsevier Inc; 2004. pp. 561-576
- [87] Guo AM, Janic B, Sheng J, Falck JR, Roman RJ, Edwards PA, et al. The cytochrome P450 4A/F-20-hydroxyeicosatetraenoic acid system: A regulator of endothelial precursor cells derived from human umbilical cord blood. *The Journal of Pharmacology and Experimental Therapeutics*. 2011;**338**(2):421-429. DOI: 10.1124/jpet.111.179036. Erratum in: *J Pharmacol Exp Ther*. 2012 Feb;**340**(2):490
- [88] Trikha M, Corringham R, Klein B, Rossi JF. Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: A review of the rationale and clinical evidence. *Clinical Cancer Research*. 2003;**9**(13):4653-4665
- [89] Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. *European Journal of Cancer*. 2005;**41**(16):2502-2512
- [90] Tchirkov A, Rolhion C, Bertrand S, Doré JF, Dubost JJ, Verrelle P. IL-6 gene amplification and expression in human glioblastomas. *British Journal of Cancer*. 2001;**85**(4):518-522
- [91] Suzuki T, Maruno M, Wada K, Kagawa N, Fujimoto Y, Hashimoto N, et al. Genetic analysis of human glioblastomas using a genomic microarray system. *Brain Tumor Pathology*. 2004;**21**(1):27-34
- [92] Saigusa K, Hashimoto N, Tsuda H, Yokoi S, Maruno M, Yoshimine T, et al. Overexpressed Skp2 within 5p amplification detected by array-based comparative genomic hybridization is associated with poor prognosis of glioblastomas. *Cancer Science*. 2005;**96**(10):676-683
- [93] Cohen JD, Javed AA, Thoburn C, Wong F, Tie J, Gibbs P, et al. Combined circulating tumour DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;**114**(38):10202-10207
- [94] Sullivan JP, Nahed BV, Madden MW, Oliveira SM, Springer S, Bhere D, et al. Brain tumor cells in circulation are enriched for mesenchymal gene expression. *Cancer Discovery*. 2014;**4**(11):1299-1309. DOI: 10.1158/2159-8290.CD-14-0471

- [95] Bednarz-Knoll N, Alix-Panabières C, Pantel K. Clinical relevance and biology of circulating tumor cells. *Breast Cancer Research*. 2011;**13**(6):228. DOI: 10.1186/bcr2940
- [96] de Gramont A, Watson S, Ellis LM, Rodón J, Tabernero J, de Gramont A, et al. Pragmatic issues in biomarker evaluation for targeted therapies in cancer. *Nature Reviews. Clinical Oncology*. 2015;**12**(4):197-212. DOI: 10.1038/nrclinonc.2014.202
- [97] Fonkem E, Lun M, Wong ET. Rare phenomenon of extracranial metastasis of glioblastoma. *Journal of Clinical Oncology*. 2011;**29**:4594-4595
- [98] Garzia L, Kijima N, Morrissey AS, De Antonellis P, Guerreiro-Stucklin A, Holgado BL, et al. A hematogenous route for medulloblastoma leptomeningeal metastases. *Cell*. 2018;**172**(5):1050, e14-1062. DOI: 10.1016/j.cell.2018.05.033
- [99] Stamatovic SM, Keep RF, Kunkel SL, Andjelkovic AV. Potential role of MCP-1 in endothelial cell tight junction 'opening': Signaling via rho and rho kinase. *Journal of Cell Science*. 2003;**116**(22):4615-4628
- [100] Gao F, Cui Y, Jiang H, Sui D, Wang Y, Jiang Z, et al. Circulating tumor cell is a common property of brain glioma and promotes the monitoring system. *Oncotarget*. 2016;**7**(44):71330-71340. DOI: 10.18632/oncotarget.11114
- [101] Bao H, Burke PA, Huang J, Chen X, Brohawn PZ, Yao Y, et al. Circulating tumor cells: Application as a biomarker for molecular characterization and predictor of survival in an all-comer solid tumor phase I clinical study. *PLoS One*. 2013;**8**(8):e58557. DOI: 10.1371/journal.pone.0058557
- [102] Gourlay J, Morokoff AP, Luwor RB, Zhu HJ, Kaye AH, Styli SS. The emergent role of exosomes in glioma. *Journal of Clinical Neuroscience*. 2017;**35**:13-23. DOI: 10.1016/j.jocn.2016.09.021
- [103] Arslantas A, Artan S, Oner U, Müslümanoğlu MH, Ozdemir M, Durmaz R, et al. Genomic alterations in low-grade, anaplastic astrocytomas and glioblastomas. *Pathology Oncology Research*. 2007;**13**(1):39-46
- [104] Katakowski M1, Buller B, Zheng X, Lu Y, Rogers T, Osobamiro O, et al. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Letters*. 2013;**335**(1):201-204. DOI: 10.1016/j.canlet.2013.02.019. [Epub 2013 Feb 16]
- [105] Hawkins BT, Davis TP. The blood-brain barrier/neurovascular unit in health and disease. *Pharmacological Reviews*. 2005;**57**(2):173-185
- [106] Malara NM, Trunzo V, Musolino G, Aprigliano S, Rotta G, Macrina L, Limongi T, Gratteri S, Di Fabrizio E, Renzulli A, Fini M, Mollace V. Soluble CD54 induces human endothelial cells ex vivo expansion useful for cardiovascular regeneration and tissue engineering application. *International Journal of Cardiology. Heart & Vasculture*. 2015;**6**(C):48-53. DOI:10.1016/j.ijcha.2015.01.004