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# **From Gliomagenesis to Multimodal Therapeutic Approaches into High-Grade Glioma Treatment**

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<http://dx.doi.org/10.5772/52782>

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## **1. Introduction**

While primary malignant brain tumors account for only 2% of all adult cancers, these neoplasms cause a disproportionate rate of cancer-related disability and death. The five-year survival rates for brain tumors are the third lowest among all types of cancer, after pancreas and lung ones, respectively.

High-grade gliomas (glioblastoma multiforme [GBM] and anaplastic astrocytoma [AA]) comprise the most common types of primary central nervous system (CNS) tumors and have a combined incidence of 5-8/100,000 population. The median survival of patients with malignant gliomas treated conservatively is 14 weeks; by surgical resection alone, 20 weeks; by surgery and radiation, 36 weeks; by the addition of chemotherapy, 40-50 weeks [1].

The oncogenesis of gliomas, in particular high-grade gliomas (HGGs), the most frequent primary malignant brain tumors in adults, is driven by several biological processes and genetic alterations, involved in the transformation of a normal cell into a neoplastic one [2, 3]. Classically this step-by-step evolution from normal cells to early-stage tumors, to aggressive cancer is found in cases of “secondary” multiforme glioblastoma (GBM), derived from a lower-grade glioma, whereas some glioblastomas occur directly without previous evidence of a lower-grade tumor. This last subset of tumors are classified as “primary” or “de novo” GBM [4]. A relatively novel feature of GBM, so as of many other types of malignant tumors, is the presence inside the gross of tumors of a particular kind of tumor cells, GBM stem cells. These cells could represent the trigger and the maintenance of gliomagenesis.

The majority of GBM cases (>90%) are primary GBM, involving mainly the elderly and genetically characterized by loss of heterozygosity (LOH) 10q [70% of cases), EGFR amplification [36%), p16<sup>INK4a</sup> deletion [31%) and PTEN mutations [25%). Secondary GBMs [5% of all cases of sporadic GBM) manifest in younger patients and are characterized often by TP53 mutations, the most frequent and detectable genetic alteration, already present at stage of low-grade astrocytomas, in 60% of cases. LOH 10q [10q25-qter) is the most frequent [70%) genetic alteration in both primary and secondary GBMs [5].

Gliomagenesis is driven by several biological processes and genetic alterations, involved in the transformation of a normal cell into a neoplastic one [2, 3].

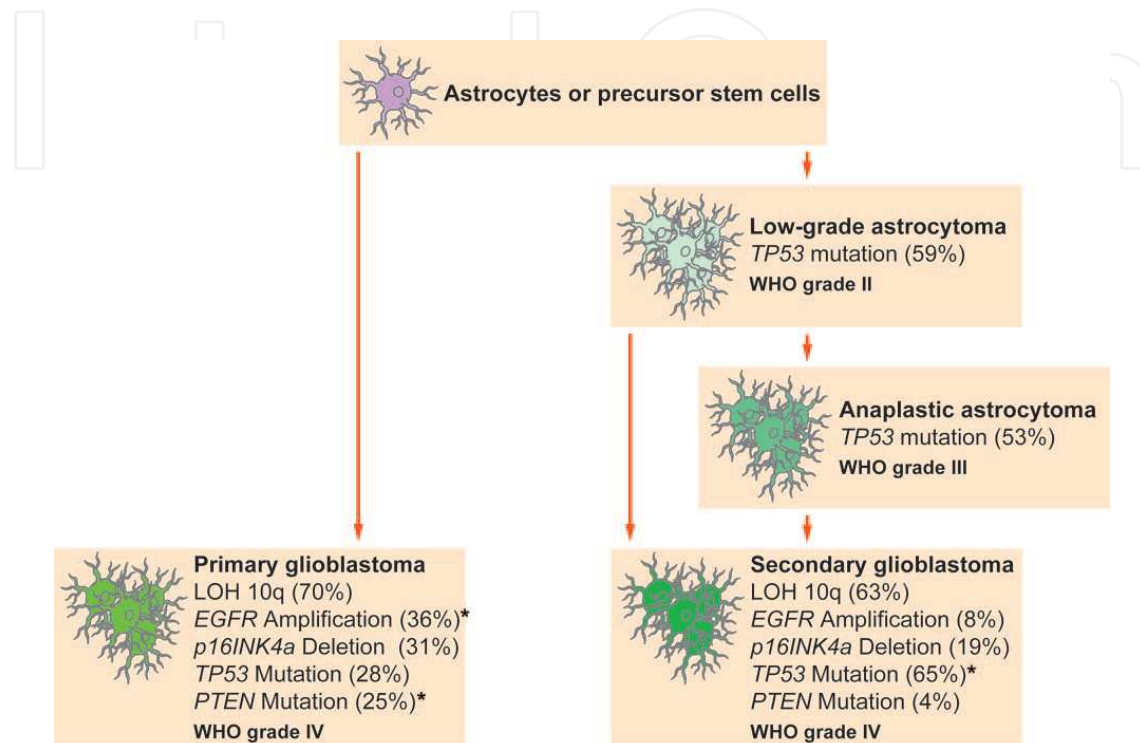
One of the most fascinating and futuristic therapeutic strategy against gliomas is represented by antisense therapy to block selectively glioma cells, trying to revert gliomagenetic molecular pathways towards the wild-type status. This aim could be reached through antisense molecules, delivered inside the brain and in particular inside tumoral cells, able to penetrate into glioma cells nucleus and to integrate with their genome to silence some specific genic functions. Among antisense molecules there are antisense oligonucleotides, ribozymes and RNA interference (iRNA). The specificity of hybridization makes antisense method an interesting strategy to selectively modulate the expression of genes involved in tumorigenesis. Glial tumors seem to be able to create a favorable environment for the invasion of glioma cells in cerebral parenchyma when they combine with the extracellular matrix via cell surface receptors. In this review we will focus on the different antisense approaches and on the mechanisms of action of each kind of antisense molecule and relative strategy in human gliomas therapy.

Among dysregulated molecular pathways, into brain tumor cells, there are activated growth factors signaling pathways, marked angiogenesis, downregulation of apoptotic genes and upregulation of antiapoptotic genes.

Gliomagenesis is driven by several biological events, such as activated growth factor receptor signaling pathways, downregulation of many apoptotic mechanisms and unbalance among proangiogenic and antiangiogenic factors. This last aspect is very crucial in glioma progression, because of prominent angiogenesis is a cardinal feature of malignant gliomas [2]. Several growth factor receptors, such epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), C-Kit, vascular endothelial growth factor receptor (VEGFR) and other growth factors receptors, are overexpressed, amplified and/or mutated in gliomas. Features of glioma cells are the loss of tumor suppressor genes, which are critical for cell growth, differentiation and function. These genes are TP53, the retinoblastoma (Rb) gene, the inhibitor of cyclin-dependent kinase 4a (INK4a) gene and the phosphatase and tensin homolog (PTEN) gene [6].

On the basis of this high importance of gene expression dysregulation inside cancer cell genome, the modulation of gene expression at more levels, such as DNA, mRNA, proteins and transduction signal pathways, may be the most effective modality to downregulate or silence some specific genic functions pathologically upregulated or introduce genes, downregulated or deleted selectively into neoplastic cells. Therapeutic genes (Transgenes) can lead to

generation of anticancer drugs within the tumor (pro-drug activation), increase of selective intratumoral drug levels without increasing systemic toxicity, protection of hematopoietic cells from drug damage by inducing drug resistance and delivery of secreted fusion proteins which combine a targeting ligand and a toxin/enzyme, sensitization of tumors to radiations, that in turn can be used to induce expression of transgenes via radiation-activated promoters [7].



(Ohgaki and Kleihues. *AJP* May 2007, Vol. 170, No. 5)

**Figure 1.** De novo vs Secondary GBM genetic pathways

Progression from low-grade to high-grade astrocytomas involve inactivating mutations of tumor-suppressor gene TP53 and elevated expression of platelet-derived growth factor (PDGF) ligands and receptors, accumulation of genetic alteration of retinoblastoma-associated cell-cycle regulatory pathways, including deletion or mutations of cyclin-dependent kinase inhibitor p16<sup>INK4A</sup>/(CDKN2A) or the retinoblastoma susceptibility locus 1 (pRB1), as well as amplification or over-expression of cyclin-dependent kinase 4 (CDK4) and human double minute 2 (HDM2). Evolution to secondary GBM is associated with deletion of chromosome 10, which includes tumor-suppressor phosphatase and tensin homolog (PTEN). The hypermethylation in the promoter region and successive mutation of p16 is evident in HGGs progression. Additionally Bcl2-like 12 increased expression (Bcl2L12) inhibits apoptosis [8].

The astrocyte elevated gene-1 (AEG-1) is over-expressed in the majority of HGGs and stimulates cellular transformation and invasion together with the Ha-ras family of retrovirus-associated DNA sequences (RAS). Furthermore, oncogenic Ha-ras induces AEG-1 expression by modulating the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway and contributes to the growth of HGGs. Mitogenetic signals activate a molecular cascade known as ras-

mitogen activated protein kinase (Ras/MAPK). MAPK inhibits the Rb gene, activates the transcriptional factor E2F and cells enter the S phase. The INK4a gene influences the Rb pathway by activating three cyclin kinase inhibitors: p15, p16, and p19. The final result is the blockage of cyclin-dependent kinase 2, 4 and 6, triggering cell-cycle progression by inhibiting pRb. Through the new technique of genome-wide sequencing and survey of gene copy number, have been discovered novel amplicons linked to glioma progression. Table 1 summarizes the main genes involved in gliomagenesis (Tab. 1).

Gene	Chromosome	Molecular alteration	Effect of the molecular alteration	Histotype (WHO Grade)
TP53	Cr17p13.1	Mutations	Cell cycle control loss, proliferation	Astrocytoma and oligodendroglioma (Grade II) – precocious mutation in secondary GBM
PDGF-A PDGFR-α	Cr4q11-q12	Overexpressio/amplification	Proliferation/invasion	Astrocytoma and oligodendroglioma (Grade II-III)
Unknown tumor suppressor genes	1p, 19q, 4q, 9p and 11p loss	Loss of Heterozygosity	Proliferation, invasiveness, angiogenesis	Astrocytoma and oligodendroglioma (Grade II-III)
Unknown tumor suppressor genes	Cr22q	Deletion	Proliferation	Astrocytoma and oligodendroglioma (Grade II)
Rb 1	Cr13q14.2	Mutations/deletion	Cell cycle control loss, proliferation	Astrocytoma and oligodendroglioma (Grade II-III)
P16	Cr9p	CDKN2/p16 deletion	Cell cycle control loss, proliferation	Astrocytoma and oligodendroglioma (Grade II-III)
PTEN	Cr10q23	LOH	Regulation Akt/PKB signaling pathway loss; proliferation and tumor growth; invasiveness, angiogenesis	Astrocytoma and oligodendroglioma (Grade III-IV)
BAX	Cr19q24	LOH	Pro-apoptotic action loss, proliferation	Astrocytoma and oligodendroglioma (Grade II-III)
EGFR (c-erb-2)	Cr7p11-p12	Amplification/overexpression	Cell transformation and proliferation	De novo GBM
MDM2	Cr12q14.3-q15	Overexpression	Cell cycle control loss and proliferation	De novo GBM

**Table 1.** Main genetic alteration in human gliomas

Gliomagenesis can develop through two different ways, resulting in evolution of a clonal genetic alteration directly from normal astrocyte to GBM or of a step by step different kinds of mutations of various genes passing gradually from normal astrocyte to low-grade glioma, to high-grade glioma, to GBM.

Primary GBM shows amplification of the epidermal growth factor receptor (EGFR), deletion or mutation of homozygous cyclin-dependent kinase (CDK) inhibitor p16<sup>INK4A</sup>/(CDKN2A), alterations in tumor suppressor PTEN on chromosome 10, and deletion in the INK4a gene with loss of p14 and p16 [6, 9]. In table 2 we, also, report the principal familial genetic syndromes associated with HGGs (Tab. 2). In the total amount of GBM, the total prevalence rate concern for the 95% cases of sporadic GBM, and only for the 5% (not more) cases of syndromic familial cancer.

Syndrome	Involved gene	Chromosomal location	Protein function	Effect of molecular alteration	Associated tumors
Turcot [Variant form of hereditary non-polyposis colorectal cancer (HNPCC) or familial adenomatous polyposis]	APC MLH1 PMS2 (mismatch repair genes) (Autosomal dominant)	5q21 3p21.3 7p22	Signal transduction, DNA mismatch repair	Hyperplasia, invasiveness and metastasis	Colorectal polyposis and primary brain tumors APC mutations are commonly associated with medulloblastoma; mismatch repair mutations are associated with glioblastoma
Gardner [Familial adenomatous polyposis, with intestinal polyposis, desmoids, osteomas, epidermoid cysts]	APC RAS DCC TP53 (Autosomal dominant)	5q21 12 18 17p13.1	Signal transduction, proliferation, cell and cell-to-matrix adhesion	Hyperplasia, invasiveness and metastasis	Colonrectal, small bowel, pancreatic, stomach, bile duct, papillary thyroid, hepatoblastoma, Brain tumors, adrenal gland cancer
Li-Fraumeny	TP53 (Autosomal dominant)	17p13.1	Transcription factor, apoptosis regulator	Abnormal protein/gain of function	Osteosarcomas, soft-tissue sarcomas, premenopausal breast, adrenal cortical, glioma, primitive neuroectodermal tumor, leukemias

Syndrome	Involved gene	Chromosomal location	Protein function	Effect of molecular alteration	Associated tumors
Von Recklinghausen Neurofibromatosis Type 1 (NF 1)	NF1 (neurofibromin) (Autosomal dominant)	17q11.2	GTPase activating protein, negative regulation of p21ras	Proliferation	Bilateral acoustic, meningioma, esp. multiple; piloid astrocytoma, mid-line glioma, diffuse glioma, intramedullary astrocytoma, spinal cord ependymoma
Neurofibromatosis Type 2 (NF 2)	NF 2	22q12.2	Cytoskeletal-cell membrane link	Proliferation	Vestibular schwannoma, peripheral schwannoma, meningioma, spinal ependymoma, astrocytoma, glial hamartoma
Tuberous sclerosis	TSC1 TSC2 (Autosomal dominant)	9q34 16p13	Not known GTPase activating protein	Cell growth and cell division control loss, proliferation	Subependymal giant cell astrocytoma, cortical tubers, cutaneous angiofibroma, peau chagrin, cardiac rhabdomyoma, adenomatous polyps of the small intestine, cysts of the lung and kidney
Retinoblastoma	RB1 (Autosomal recessive)	13q14	Regulator of transcription factors	Proliferation	Retinoblastoma, pineoblastoma, glioma, osteosarcoma
Multiple hamartoma (Cowden Disease)	APC hMLH1 hMSH2 PMS2 PTEN (Autosomal dominant)	5q21 3p21.3 2p22-21 7p22 10q23.3	Signal transduction, DNA mismatch repair	Hyperplasia; proliferation, apoptotic cell death loss, invasiveness and metastasis	Dysplastic cerebellar gangliocytoma, hamartomas (skin, breast, thyroid, colon, intestines, mouth)



Syndrome	Involved gene	Chromosomal location	Protein function	Effect of molecular alteration	Associated tumors
Gorlin	PTCH1 PTCH2 (Autosomal dominant)	9q22.3-q31 1p32	Transmembrane receptor	Proliferation	Medulloblastoma, meningioma, astrocytoma, multiple basal cell carcinomas, palmar and plantar pits

**Table 2.** Principal familial genetic syndromes associated with human gliomas

## 2. Glioma invasion

Glioma cells invasion consist of an active translocation of glioma cells through host cellular and extracellular matrix barriers [10].

Three steps are fundamental in the phenomenon of glioma invasion: a) adhesion of glioma cells to proteins of the surrounding extracellular matrix (ECM) mediated by cell adhesion receptors; b) degradation of ECM components by proteases secretion by glioma cells; c) migration of glioma cells into the newly created space through the ECM.

ECM is composed of proteoglycans, glycoproteins, collagens and also contains fibronectin, laminin, tenascin, hyaluronic acid, vitronectin. In recent years the glioma invasion has also been interpreted by numerous authors in terms of interaction between neoplastic cells and ECM [11].

Critical factors include the synthesis and deposition of ECM components by glioma and mesenchymal cells, the release of ECM-degrading activities for remodeling interstitial spaces, the presence of adhesion molecules (matrix receptors on glioma cell surfaces that specifically recognize and adhere to ECM components) and the effects of cell-matrix interactions on the behavior of glioma cells. ECM modification aids the loss of contact inhibition allowing tumor cells to freely migrate and invade the surrounding tissues. Changes in these ECM components are felt to modulate brain tumor growth, proliferation and invasion, although specific interactions and exact mechanisms are unknown.

Integrins, a class of adhesion molecules, play a major role in glioma cell-matrix adhesion. Integrins regulate many aspects of the cell behavior including survival, proliferation, migration and differentiation. They act with a physical trans-membrane link between the ECM and the cytoskeleton and with a bi-directional signal across the cell membrane. Integrins of the  $\beta 1$  and  $\alpha v$  classes are expressed on different cell types, including neurons, glial cells, meningeal and endothelial cells.  $\beta 2$  integrins are specifically expressed by leukocytes and they are found on microglia and on infiltrating leukocytes within the CNS. Down-regulation of  $\beta 1$  expression in intracerebrally transplanted glioma cells in vivo by placing  $\beta 1$  antisense sequences under an inducible, tetracycline-responsive promoter markedly inhibited diffuse brain invasion [13-17].



Down-regulated  $\beta 1$  integrin protein levels in vivo probably affect interactions of glioma cells with ECM components, leading to reduced migration along vascular basement membranes.

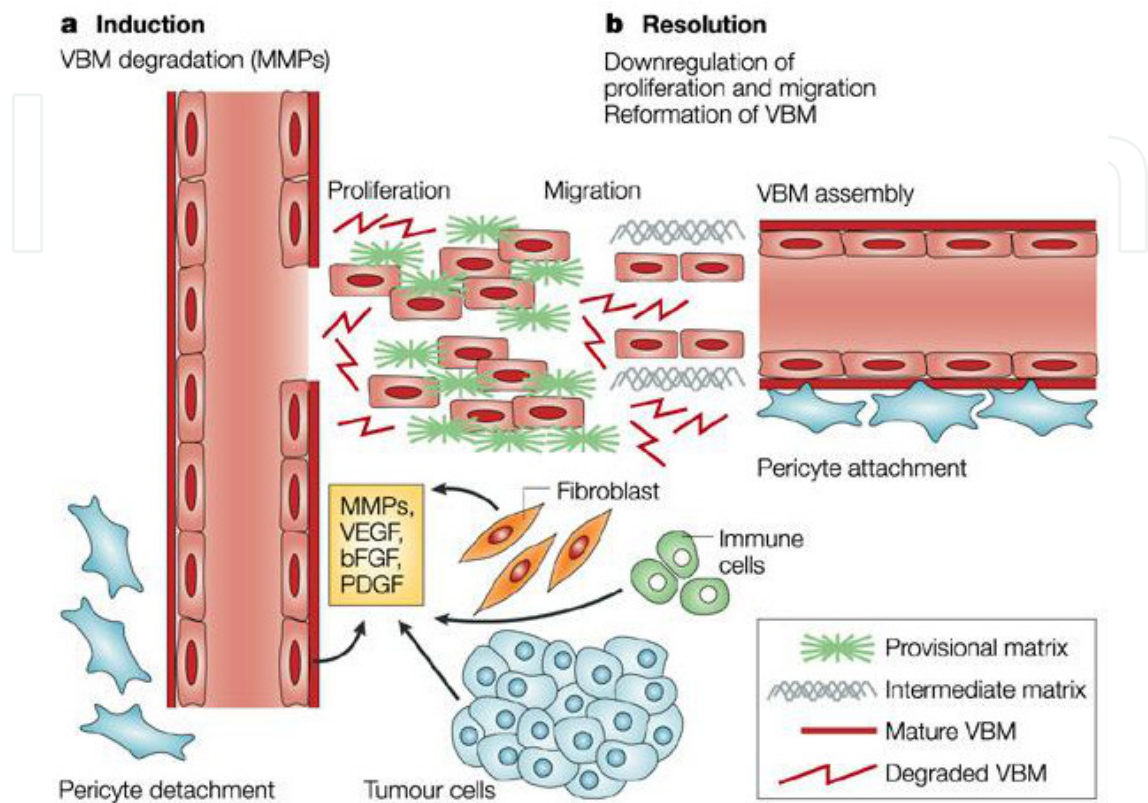


Figure 2.

The proteolytic degradation of the basement membrane (BM) is mediated by proteases, such as the matrix metalloproteases (MMPs), secreted by tumor and stromal cells [11, 12]. MMPs are secreted as proenzymes and are activated by proteolytic cleavage of their amino-terminal domain. MMPs play an important role in human brain tumor invasion, probably due to an imbalance between the production of MMPs and tissue inhibitor of metalloproteases-1 (TIMP-1) by the tumor cells. Among these molecules, MMP-1 is the crucial enzyme able to initiate breakdown of the interstitial collagens, collagen type 1, collagen type 2 and collagen type 3; in this way it activates the other MMPs which allow the glioma cells to infiltrate normal brain tissue.

### 3. Angiogenesis

The formation of new blood vessels from existing microvessels is angiogenesis, an histological indicator of the degree of malignancy and prognosis of patients. Of all solid tumors, malignant brain tumors show the highest degree of vascular proliferation. The WHO classification distinguishes low grade from high grade diffuse astrocytomas by the presence of microvascular proliferation as a diagnostic criterion and an independent prognostic parameter [18].

HGGs are characterized by extensive microvascular proliferation and a higher degree of vasculature. The presence of marked endothelial glomeruloid-like proliferations demonstrates active tumor invasiveness through vascular angiogenesis, disruption of pre-existing anatomical structures and neoplastic cellular migration along neoangiogenic vascular channel and through the BM, with evidence of proliferation of new fine capillaries [8, 19]. These mechanisms demonstrate a very strong and bidirectional correlation between glioma angiogenesis and invasiveness into glioma progression. The new vessel growth is stimulated through the tumor cells secretion of pro-angiogenic growth factors; these factors bind to receptors on endothelial cells thereby activating them [20].

Vascular endothelial growth factor-A (VEGF-A) is regulated in HGGs and it is secreted by tumor cells as well as by stromal and inflammatory cells. VEGF-A can be linked in the ECM through the interaction with proteoglycans or glycosaminoglycans. The expression of the receptors VEGFR1 and VEGFR2 is regulated on the endothelial cells in HGGs. The ligands for VEGF3 (VEGF-C&D) are expressed by multiple cell types that surround the angiogenic vessels, suggesting the existence of a novel pro-angiogenic paracrine signaling pathways in these neoplasms [10, 21]. Basic fibroblast growth factor (bFGF) is expressed by vascular cells and, focally, by the tumor cells too. The receptors for bFGF include FGFR1, expressed by both the tumor cells and the tumor endothelial cells; FGFR2 is expressed only by the tumor cells whereas FGFR4 is not detected in HGGs [22]. The binding of VEGF on endothelial cells activates the phosphatidylinositol-3-hydroxyl kinase (PI3K)/protein kinase B (Akt) pathway, whereas the bFGF receptors are predominantly shown through the protein kinase C $\alpha$  (PKC $\alpha$ ) pathway. The activation of endothelial cells results in increased expression of cell adhesion receptors, such as integrins  $\alpha$ v $\beta$ 3 and  $\alpha$ 5 $\beta$ 1, and in increased cell survival, proliferation and migration responses. Other pro-angiogenic growth factors, including interleukin-8, hepatocyte growth factor, urokinase can promote or amplify angiogenesis.

Cancer angiogenesis is similar to angiogenesis seen during development except that it is less controlled and precise [23]. These blood vessels have many marked features including blind ends, erratic size and connections, and most significantly, large gaps in the endothelial lining. This last feature is important because it signifies that larger molecules can enter the tumor effectively bypassing the blood brain barrier. In fact, tumor vasculature can have intercellular gaps as large as 2  $\mu$ m which is large enough for passive accumulation of 100-200nm particles into the tumor interstitium [24]. Neovascularization in brain tumors correlates directly with their biological aggressiveness, degree of malignancy and clinical recurrence and inversely with the post-operative survival of patients with gliomas. Diffuse astrocytomas tend to progress from grade II to grade III tumors with a time interval of several years, whereas, progression of grade III to grade IV is more rapid, typically 2 years. GBMs that arise from a low-grade glioma lesion are called "secondary glioblastoma".

However, in most cases, GBMs appear "de novo" and are thus termed "primary glioblastoma". Regardless of their mode of progression, primary and secondary GBMs are morphologically indistinguishable and show their histologic hallmarks, such as "glomeruloid" microvascular tufts and necrosis [25, 26].

The discovery of hypoxia inducible factor-1 (HIF-1) and the observation that hypoxia-induced HIF-1 $\alpha$  expression in pseudopalisading cells, into intratumoral necrotic areas, was concomitant with the expression of one of its target genes, VEGF, established a biological link between hypoxia and angiogenesis [27]. The formation of new blood vessels occurs physiologically during embryogenesis. In adult life it is observed in the female reproductive system and during wound healing and in a wide range of pathologic settings, such as ischemic diseases, chronic inflammatory reactions, and neoplasia. During embryonic development, blood vessels are newly formed from endothelial precursors and hematopoietic stem cells, a process known as vasculogenesis [28]. In contrast, angiogenesis, the sprouting of new blood vessels from pre-existing ones results from an altered balance of proangiogenic factors and antiangiogenic factors [29, 10].

The sequence of events leading to the formation of new blood vessels is well characterized and involves an initial VEGF-mediated increase of vascular permeability leading to extravasation of plasma proteins associated with dilatation of native vessels and reduction in their pericyte coverage. Subsequently, endothelial cells migrate and proliferate. For this cascade to occur, deposition of a proangiogenic matrix for the newly sprouting vessel is essential. This involves breakdown of the vascular basement membrane and extracellular matrix (ECM) through the action of cathepsin B, matrix metalloproteases (MMPs) and other enzymes as well as the expression of matrix proteins such as fibronectin, laminin, tenascin-C and vitronectin. Finally, the angiogenic process culminates in the assembly of endothelial cells to form a vascular lumen followed by the elaboration of a new basement membrane and the recruitment of pericytes. In contrast to the accepted dogma that tumor development occurs in 2 phases (avascular and vascular), we observed that tumor growth in the brain follows 2 vascular phases. In the first vascular phase, the vessels are native cerebral vessels, which are coopted by tumor cells, while in the second phase, there is true neovascularization arising from existing vessels. During the transition period between these two phases, hypoxia driven HIF-1 expression occurs which results in VEGF secretion and induction of neovascularization. In Stage IV, angiogenesis adjacent to the necrotic area is triggered in response to increased expression of HIF-1 $\alpha$  and VEGF, a process that rescues the remaining tumor cells. Thus, it is possible to suggest four sequential steps in glioma progression: i) perivascular organization, ii) proliferation, iii) vascular regression followed by necrosis, and iv) angiogenesis.

Glioma vasculature is structurally and functionally abnormal and it correlates and leads to vasogenic edema, increased interstitial pressure, and heterogeneous delivery of oxygen and drugs [23].

VEGF expression is stimulated by hypoxia and acidosis, and probably correlates with many other growth factors and their specific receptors (EGFR, HGFR, PDGFR, C-Kit, IGFR), and downstream signaling pathways (PI3K-Akt, Ras-MAPK) upregulation and activation in gliomas.

Many other proangiogenic factors are upregulated in gliomas and this aspect might explain the failure of many actual antiangiogenic therapeutic strategies in gliomas management. Among these other angiogenic factors a very important role has been absolved by bFGF, IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and stromal-cell-derived factor (SDF)-1  $\alpha$  [2].

Another group of endothelial growth factors is represented by angiopoietins. Their signal transduction pathway passes via the Tie2 receptor tyrosine kinase expressed on endothelial cells. In particular, Ang-1 and -2, have been implicated in glioma angiogenesis. Ang-1 mediated activation of Tie2 is required for stabilization, remodelling and maturation of blood vessels, promotes angiogenesis and tumor growth and is associated with an increased number of highly branched vessels covered by pericytes. While VEGF and Ang-1 may act in concert (proliferation and maturation), Ang-2 has been implicated in further remodeling of the initial microvasculature [30, 31]. Increased expression of Ang-2 on GBM microvasculature appears early during glioma angiogenesis. However, binding of Ang-2 to the Tie2 receptor on endothelial cells antagonizes this receptor's phosphorylation, thereby disrupting contacts between endothelial and periendothelial support cells and disengaging pericytes from the tumor vessels during initiation of vessel sprouting or regression. Examination of the expression patterns of angiopoietins and their receptors suggests a role in GBM vasculature and malignant transformation. For example, increased Tie2 expression has been observed with increasing human astrocytoma grading. Ang-2 and Tie2 expression are absent in the normal brain vasculature but are induced in tumor endothelium of coopted tumor vessels prior to their regression. Of particular importance, treatment of glioma cell derived mouse xenografts with a dominant negative form of Tie2 results in a significant decrease in tumor growth [32].

A new more complex understatement of angiogenic pathways in glioma progression gives a crucial role to the  $\text{Ca}^{2+}$  permeable transient receptor potential (TRPC) channels in tumor angiogenesis, and in particular in endothelial permeability up-regulation, focal adhesion assembly, cell orientation and directional migration and involvement in hypoxia-induced angiogenesis and vascular remodeling. According this complex molecular pathological network, tumor angiogenesis should be not only the result of physiological adaptation to hypoxia in response to an increasing tumor mass but it should be also the result of critical genetic mutations that activate a transcriptional program for angiogenesis. Calcium ( $\text{Ca}^{2+}$ ) is an important second messenger and its entry through plasma membrane affects angiogenesis. Several reports indicate that  $\text{Ca}^{2+}$  permeable transient receptor potential (TRP) channels belonging to the TRPC, TRPV and TRPM represent target genes, down-stream to Notch1, PTEN, NFAT and Hif-1 transcriptional factors are activated during tumor angiogenesis. Several studies indicate that angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) may activate TRP channels at transcriptional and post-transcriptional levels, causing a subsequent rise in endothelial  $[\text{Ca}^{2+}]_i$ , which modulates signal transduction pathways regulating the angiogenesis. Conversely,  $\text{Ca}^{2+}$  influx through TRP channel activation may stimulate endothelial cells to trigger transcription, production and release of angiogenic growth factors such as VEGF and platelet-derived growth factor (PDGF) that stimulate angiogenesis.

In the field of new targeted-based molecular therapeutic approaches, TRP channels, and not only VEGF/VEGFR and HIF-1 $\alpha$ , might be a promising target for new therapeutic agents to anti-angiogenic therapy [33].



## 4. Current treatment of glioblastoma

The efficacy of current anti-cancer multimodal therapeutic strategies in gliomas is limited by the lack of specific therapies against malignant cells, and the prognosis in patients affected by primary brain tumors is still very unfavorable. Glial tumors seem to be able to create a favorable environment for the invasion of neoplastic cells when they combine with the extracellular matrix via cell surface receptors, through the upregulation of crucial pathways such as angiogenesis and invasion. The major problem in brain drug delivery is the presence of the blood brain barrier which limits the delivery of many chemotherapeutic agents and other kinds of therapeutic molecules. This event often contributes to the failure of treatment. Current treatment of glioblastoma (GBM) is difficult and a great mysterious challenge, and, results in high recurrence rates. Upon display of symptoms of a brain tumor (seizure, chronic headache, loss of consciousness, loss of motor/sensory function) a patient will undergo MRI for detection of the tumor. This is followed by a host of treatments to remove the bulk of the tumor if possible, starting always when possible from gross total removal of tumor [34]. Patients are then monitored for recurrence. This is standard protocol for treatment of glioma regardless of grade [35]. This line of treatment can often lead to decreased quality of life. Last, if tumor recurs, there are last line treatments given after which there is no ability to treat further. The treatment of glioma is varied depending on grade, but this discussion will focus on the treatment of the most aggressive form: glioblastoma (GBM). The common treatment after detection has several components including: 1. Surgical resection of tumor from the primary site, 2. Lining of the primary site with carmustine wafers designed to kill cells in the close proximity, after gross total removal and histological confirmation of high-grade gliomas 3. Metronomic oral temozolomide chemotherapy and 4. Radiotherapy to the brain. As mentioned previously, in the case of recurrence, this treatment is coupled with anti-angiogenic therapy to prolong patients' life.

## 5. Surgical resection

Represents the microneurosurgical removal of the tumor from the primary site. The general procedure involves craniotomy followed by suction and gross total removal of tumor from the site. Though there are experimental imaging agents, such as optically visible fluorescent dyes coupled with intravital magnetic resonance imaging of the brain for guidance and assessment of accuracy and completion, this is not an exact science [35-37].

## 6. Local, intracavitary, chemotherapy

It is applied in the resection site after removal of the tumor gross in the form of Poly (lactic-co-glycolic acid)-BCNU wafers that slowly degrade and release the chemotherapeutic BCNU (Carmustine - gliadel®) into the resection site, after extemporaneous histological intra-

operatory examination and confirmation of high-grade gliomas [38]. This local chemotherapeutic approach has been reported to yield a slight increase in survival times, as opposed to leaving the tumor site empty, however, it still does not allow adequate decrease in recurrence. Use of gliadel® has become less common place due to both cost-benefit analysis and preference of use by surgeons [39, 40].

## 7. Radiotherapy and stereotactic radiosurgery

It involves the use of gamma-irradiation in order to induce cell death through DNA damage, often inside a post-operative adjuvant therapy. The technologic support to apply this therapeutic step does see the use of Gamma-knife, cyber-knife or conformational collimation system-based linear accelerator. This therapy is used in over 50% of all cancer patients at some point during treatment and has been in use in cancer for almost a century. In brain tumors, this treatment works by sending gamma rays through to the site of the tumor to directly damage DNA, but in longer-lasting effects, creates free radicals which can go on to cause further damage [41, 42]. Radiation therapy involves not just radiation of the tumor, as would be desirable, but irradiation of an area which can include both tumor tissue and healthy brain tissue. Radiotherapy does usually results in death of large amounts of the tumor, however, recent discoveries suggest that radiation may actually be causing side effects which can aid in recurrence. Glioma cells exposed to radiation *in vitro* show increased invasion post treatment, indicating progression and enhanced malignancy of disease [43]. Further, radiation leads to many side effects associated with cancer malignancy and disease progression, including the creation of free radical species, degradation of the extracellular matrix, inflammation and immune cells recruitment, and increased blood flow to the tumor area [44, 45].

Radiotherapy improved several clinical end points in brain tumors. Standard radiation therapy of gliomas involves the provision of 59.4 / 60 Gy, in 30/33 from 1.8 to 2 Gy daily fractions, and each should begin within 4-6 weeks after surgery. Treatment is generally given to the area of the tumor and a surrounding margin ("limited-field" method). Postoperative radiotherapy significantly prolongs median survival to approximately 12 months for glioblastoma multiforme and to 36 months for anaplastic astrocytoma [46]. A slight improvement of the results obtained with conventional radiotherapy has been achieved through the hyperfractionation daily, the use of radiosensitizers or hyperoxygenation by hyperbaric treatment [47].

Stereotactic radiotherapy is helpful for the treatment of high grade gliomas and involves the local system of radioactive sources (interstitial brachytherapy) or external targeted radiotherapy. In case of tumors of a limited volume boost stereotactic radiotherapy, administered by linear accelerator or Gamma-Knife or Cyber-knife and performed after limited-field radiotherapy, achieved a slight increase in survival in some studies [48].

The metabolic radioimmunotherapy, using radiolabeled antibodies injected systemically or intramuscularly, is still experimental because there are no phase III trials that showed a survival advantage [49].



## 8. Systemic chemotherapy

At present there is not a standard treatment of anaplastic oligoastrocytoma and oligodendroglioma but the common practice, based on recommendations of the National Comprehensive Cancer Network (NCCN), consists in surgery followed by radiation therapy. Adjuvant chemotherapy can also be administered and consists in PCV or TMZ.

Loss of heterozygosity (LOH) of chromosome 1p/19q (present in approximately 40-60% of oligodendroglial tumors) has been showed to be related to the sensitivity to the treatment with alkylating agents (especially TMZ and poli-chemotherapy scheme PVC) and to a more favorable prognosis. For a most appropriate therapy would therefore be necessary to look for the loss of heterozygosity 1p and 19q in all tumors with oligodendroglial component [50].

Two clinical trials are evaluating the role of chemotherapy in the treatment of anaplastic glioma correlating it to the co-deletion 1p/19q. The first, EORTC 26053-22054 is a Phase III trial on concurrent and adjuvant temozolomide chemotherapy in non-1p/19q deleted anaplastic glioma. The second is EORTC 26081-22086 a Phase III intergroup study of radiotherapy versus temozolomide alone versus radiotherapy with concomitant and adjuvant temozolomide for patients with 1p/19q codeleted anaplastic glioma.

Oligodendrogliomas and oligoastrocytomas, especially in anaplastic forms, are highly sensitive to adjuvant chemotherapy with PCV after radiation therapy, with a significant increase in progression-free survival (23 months vs. 13 months) compared to radiotherapy alone. An increase of the progression-free survival is also reflected by administering the PCV before radiotherapy. None prolongation of the median overall survival time is noted [51]. Nevertheless, there is the common practice of proposing chemotherapy as the initial therapy for pure oligodendroglioma. Further, in the case of small tumors RT remains the best therapeutic choice. Although there is no phase III clinical studies comparing PCV and TMZ in clinical practice TMZ is preferred as first line treatment for the lower toxicity and comparable efficacy to PCV. Second line chemotherapy with TMZ in recurrent oligodendroglial tumors after PCV-chemotherapy gave excellent results, and similar responses to PCV but with less toxicity [52].

Standard therapy of anaplastic astrocytoma, according to Stupp protocol, is radiotherapy with adjuvant TMZ, administered for the duration of radiotherapy ( 75 mg/m<sup>2</sup> daily) and after in six cycles (150-200 mg/m<sup>2</sup> for five days every 28 days). There are no randomized studies with adequate statistical power in patients with anaplastic astrocytoma that demonstrated a benefit of adjuvant or concomitant chemotherapy with nitrosoureas. In a EORTC study, comparing adjuvant dibromodulcitol and BCNU chemotherapy with radiotherapy alone in patients with anaplastic astrocytoma, no statistically significant improvement in survival was observed after BCNU/DBD adjuvant chemotherapy [53]. Temozolomide, an imidazotetrazinone acting through the formation of a reactive methyldiazonium cation and methylation of O6-guanine in DNA, induce its clinical effects through the activity of O6-alkylguanine-DNA alkyltransferase (AGT), a DNA repair protein that removes O6-alkylguanine adducts in DNA, result of translation from gene MGMT, whose grade of promoter

methylation is recognized important epigenetic predictive factor to temozolomide therapeutic response. The therapeutic advantage is showed for patients with MGMT (methyl-guanine methyltransferase) methylated tumors. The enzyme remove alkyl groups from the O<sup>6</sup> position of guanine, one of the targets of alkylating agents. Epigenetic silencing of the MGMT gene in tumor tissue occurs through the methylation of the CpG islands in the promoter region. Patients whose tumors have MGMT promoter methylation are likely to benefit from the addition of TMZ chemotherapy [54]. TMZ is characterized from an excellent oral bioavailability and good penetration of the blood-brain barrier (BBB). The activity of temozolomide is highly dependent on dosing schedule, with multiple administrations being more effective than a single dose (as normally codified in Stupp protocol). Peak plasma concentration is achieved within 30-60 min of oral administration and the compound has an elimination half-life of one to two hours. Myelosuppression, which is dose limiting at 1,200 mg/m<sup>2</sup>, and nausea and vomiting are the most frequent adverse events [55].

A new promising agent, recently evaluated for the treatment of anaplastic astrocytoma, is Trabedersen, a phosphorothioate antisense oligonucleotide that act as an inhibitor of TGF-  $\beta$ 2 (Transforming growth factor-beta 2) biosintesis. TGF-  $\beta$ 2 levels seems to be higher in high grade gliomas. TGF- $\beta$ 2 is an attractive target because it regulates key mechanisms of carcinogenesis, in particular immunosuppression and metastasis. The compound was tested in phase I/II studies, in which it was administered at two doses (10  $\mu$ M or 80  $\mu$ M) comparing response rate, survival, and safety respect to standard chemotherapy (TMZ or PCV). Trabedersen 10  $\mu$ mol/L was superior to trabedersen 80  $\mu$ mol/L in both efficacy and safety for high-grade gliomas, especially in anaplastic astrocytoma. In patients with anaplastic astrocytoma, the trabedersen 10  $\mu$ mol/L group had a higher overall survival rate at 24 months than the trabedersen 80  $\mu$ mol/L group and the control group (83.3%, 53.3% and 41.7%, respectively). The median overall survival for patients with anaplastic astrocytoma was higher in both trabedersen groups than in the chemotherapy control group, with a survival benefit for 10  $\mu$ mol/L trabedersen over chemotherapy (17.4 months). Based on the promising results of these studies, presented at the 101st annual meeting of American Association of Cancer Research, in 2010 started the pivotal Phase III study SAPPHIRE (Efficacy and Safety of AP 12009 in Adult Patients With Recurrent or Refractory Anaplastic Astrocytoma [WHO Grade 3] as Compared to Standard Treatment with Temozolomide or BCNU: A Randomized, Actively Controlled, Open label Clinical Phase 3 Study). Unfortunately, the study has been stopped prematurely in January 2012, due to slow patient recruitment [56].

Historically, the standard of care for older adult with GBM was surgical resection or biopsy, followed by involved-field radiotherapy. The new therapeutic standard is based on the results of several studies that demonstrated a significant survival benefit in GBM patients with the addition of concurrent and adjuvant TMZ to radiation. Patients treated with TMZ/RT had a median survival time of 15 months, compared with 12 months for the patients initially treated with RT alone. Further, the first group showed a 2-year survival rate of 26% compared with only 10% for the RT group. Now the standard treatment consist in surgery followed by RT with adjuvant TMZ, administered for the duration of radiotherapy and after in six cycles (Stupp protocol) [57].

A therapeutic alternative or adjunctive treatment is BCNU wafer (Gliadel®), positioned at surgery and followed by radiation therapy. Gliadel® is a biodegradable polymer wafer impregnated with BCNU. At the time of resection, up to eight wafers are implanted into the surgical cavity. Water in the interstitial fluid causes the polymer slowly to degrade. The BCNU is thus released in a controlled manner over several days to weeks and diffuses into the brain parenchyma at a high dose density. It causes a statistically significant increase in survival compared to patients treated only with surgery and radiotherapy. The SIGMA (Stupp including Gliadel for glioma) is a multi-center, spontaneous observational trial to evaluate the efficacy of Gliadel® wafers plus concomitant TMZ in patients with newly-diagnosed high grade glioma. This study is conducted at the Foundation of the Carlo Besta Neurological Institute, IRCCS, in Milan and is based on several examples in literature on this association. Samaggi et al. of the IRCCS published a study on the research and comparison of data in literature about efficacy and toxicity in patients with newly diagnosed GBM treated with the combination of Gliadel® and the Stupp protocol. Analysis of data revealed that this combination is well tolerated and it significantly improved survival without a substantial increase in toxicity [58].

Recently was demonstrated that for elderly patients with newly diagnosed glioblastoma, therapy with TMZ alone is an alternative to radiotherapy. In the study, published in the July issue of the *Lancet Oncology*, were recruited 412 older patients. 195 were randomly assigned to TMZ and 178 to radiotherapy. The results showed that single-agent TMZ and radiotherapy alone seem equally effective. Both treatments were well tolerated. Major side effects observed were neutropenia, (16 in the TMZ group and 2 in the RT group), lymphocytopenia (46 vs. 1), thrombocytopenia (14 vs. 4), hepatic enzyme increased (30 vs. 16), infections (35 vs. 23) and thromboembolic events (24 vs. 8). Even in older patients the MGMT methylation status of tumor is a predictive marker for the success of therapy with alkylating agents as mentioned above. So testing of tumor MGMT methylation status in elderly patients with newly diagnosed glioblastoma should be a priority to decide the best treatment [59].

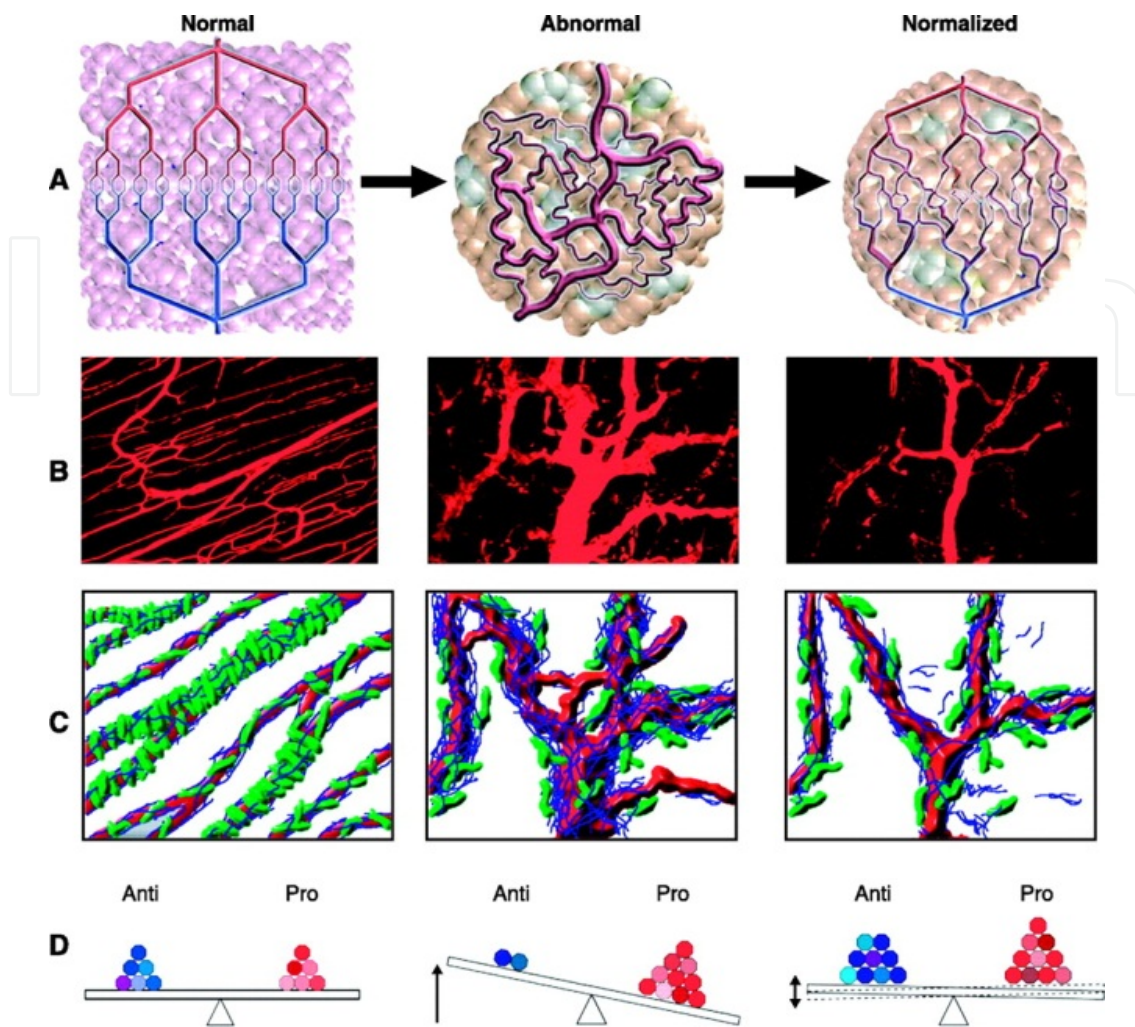
## 9. Antiangiogenic therapy

Angiogenesis inhibition represents a new target for therapeutic intervention in malignant gliomas because GBMs are highly vascularized brain tumors and their growth seems to be angiogenesis dependent.

The inhibition of angiogenesis is achieved through the use of inhibitors of VEGF receptor (such as bevacizumab or aflibercept) or kinase or integrin inhibitors (cilengitide or erlotinib) which have been tested in several clinical trials.

The antiangiogenic effect is achieved through different mechanisms assumed on the basis of results from several studies. According to the first hypothesis (starvation hypothesis), the reduction of new blood vessels formation decreases the amount of metabolites that may reach the tumor, causing tumor cell death. According to a new hypothesis (normalization hypothesis), in the early stages (1 month) of the antiangiogenic therapy there is a normalization of the blood vessels, followed by their damage due to hypoxia, and then by the reduction of tumor growth.





Goel S et al. *Physiol Rev* 2011

**Figure 3.**

These hypothesis have been confirmed by recent studies in which was highlighted the increase of metabolites associated with anaerobic glycolysis in the long-term therapy with anti-VEGF. This would explain the failures obtained in the treatment of solid tumors with anti-VEGF monotherapy but also the excellent results of several trials with the association anti-angiogenic agent and chemotherapy, even compared to chemotherapy alone [60].

The need to associate an antiangiogenic agent to traditional therapy is useful not only to improve the vehiculation of the anticancer drug but also to control recurrences. According to clinical experience, after surgical resection, radiotherapy and adjuvant chemotherapy with TMZ, there is a recurrence or progression within six months. This recurrence is localized in most cases where there was the resection of the primary tumor, in which are localized tumor cells infiltrated. This phenomenon has been explained assuming that the primary tumor secretes not only pro-angiogenic agents but also anti-angiogenic agents that inhibit neovascularization at the level of infiltrating tumor cells, which are located at a certain distance from

the primary tumor. For this reason the use of anti-angiogenic agents is recommended not only in newly diagnosed gliomas but also in recurrences [61].

## 10. Anti VEGFR — Bevacizumab

Bevacizumab (Avastin®; Genentech, CA, USA) is a recombinant humanized monoclonal antibody that binds with high affinity to human VEGF preventing it from binding to its receptors VEGFR1 and VEGFR2, on the surface of endothelial cells and thereby determining the block of the cascade of events following its receptors activation. Will be therefore inhibited proliferation and increased permeability due to receptor VEGFR2 and tumor growth due to receptor VEGFR1 through the production of matrix metalloproteinases. Bevacizumab inhibits the growth of new blood vessels and reduces the blood flow to the tumor. Like other antiangiogenic agents it shows, in the initial phase of the treatment, a normalizing effect on blood vessels. This normalizing effect is important because tumor vasculature is often incomplete and tends to leak. The abnormal permeability may prevent the administration of cancer treatment such as chemotherapy. Bevacizumab can normalize the chaotic vascularization of the tumor, in order to maximize the effectiveness of the overall therapeutic strategy.

In the U.S., Avastin® is the first anti-angiogenesis therapy approved by Food and Drug Administration (FDA) to treat patients with glioblastoma multiforme (GBM) when this form of brain cancer continues to progress following standard therapy. The drug is also approved for the treatment of advanced stages of other 3 types of cancer: breast, colorectal, and non small cell lung cancer (NSCLC). Even before FDA approval, bevacizumab was widely used for this indication in the United States and in some European countries [62].

Many clinical trials evaluated efficacy of bevacizumab and irinotecan versus bevacizumab alone (and the addition of irinotecan at progression) with several results but also with many questions unanswered about the end points used, the increase in survival, the best dose and timing. Teri N. Kreisl et al. conducted a trial to evaluate single-agent activity of bevacizumab in patients with recurrent glioblastoma. The dose of bevacizumab was 10 mg/kg every 2 weeks. At tumor progression bevacizumab was associated in combination with irinotecan 340 mg/m<sup>2</sup> or 12 mg/m<sup>2</sup> every two weeks. The study found that single-agent bevacizumab has a significant antitumor effect in recurrent glioblastoma and show less overall toxicity but also a lower response rates (29% vs. 46%) compared with combination therapy. The study shows that it is not always strictly necessary the addition of irinotecan after tumor progression during the treatment with bevacizumab. It is not possible, however, exclude the contribution of irinotecan if the two agents are used together in the initial treatment. Will be necessary to conduct further studies to understand whether the addition of this drug is so useful to offset the toxic effects associated with its use [63].

Results from this trial are consistent with data presented by Vrendenburgh et al. emerged from a phase II trial testing bevacizumab plus irinotecan in recurrent GBM. They reported a response rate of 57% and a 6-month progression-free survival of 46% with this combination. The rationale of combining the two drug is based on the normalization effect of antiangiogenic

agents that reduce the intratumoral pressure and increase drug delivery to tumor. But there could be also another unwanted effect, the reduction of drug penetration to the brain due to the restoring of the blood-brain barrier [64].

Avastin® is approved in Europe for the treatment of advanced stages of four types of cancer: colorectal cancer, breast cancer, lung cancer and kidney cancer. Together, these four types of cancer cause nearly 3 million deaths every year. The use of the drug in Italy for malignant gliomas is possible through the enrollment in clinical trials or the compassionate use. Compassionate use programs are intended to facilitate the availability to patients of new treatment options under development. National compassionate use programs, making medicinal products available either on a named patient basis or to cohorts of patients, are governed by individual Member States legislation. In Italy the global compassionate use is ruled by the Ministerial Decree dated 8th May 2003. Drugs under experimentation may be used outside the experimentation if there isn't a valid therapeutic alternative [65].

The neuro-oncology department of Besta Institute is conducting a study on the therapeutic use of bevacizumab and irinotecan in patients with recurrent grade III and IV glioma in the absence of therapeutic alternative since is emerged by various studies the effectiveness of this association [66]. However, not all patients respond to bevacizumab-irinotecan combination. This is probably due to the different pathways involved in tumor angiogenesis, in which VEGF is only one factor, even though prevalent. The lack of response in some patients may be related to any of the alternative routes [67].

Despite their contribution in the treatment of vascularized GBM, these agents have some serious side effects including hypertension. For this reason, this therapy is a problem for patients with underlying hypertension. In such cases is possible to associate calcium channel blockers or ACE inhibitors to cancer therapy. ACE inhibitors are often preferred because in hypertension caused by VEGF inhibitors the way of NO is involved, and it can be blocked by these antihypertensive agents. Another side effect caused by the antiangiogenic therapy is proteinuria, which can be prevented by these agents. The bevacizumab-irinotecan combination is responsible for serious side effects such as renal failure and gastric perforation, which in some cases lead to discontinuation of treatment. It is recommended that bevacizumab not be initiated for at least 28 days after surgery and until any surgical wound is fully healed and that bevacizumab be discontinued at least 28 days prior to elective surgery [62].

## 11. Integrin inhibitors — CILENGITIDE

Integrins are a family of transmembrane receptors whose natural ligands are represented by vitronectin, fibronectin, laminin. Their ligands are recognized by the common portion arg-gly-asp (RGD); upon binding occurs a conformational change, the formation of clusters of integrins and the activation of intracellular kinases, from which depend many intracellular signal cascades. These cascades result in the processes of proliferation, differentiation, motility, survival and cell adhesion. Some integrins are ubiquitous, others are specific for various tissues and are also expressed on the surface of different types of tumors. In such



cases there will be an increase in the levels of both integrins in tumor cells than in endothelial cells in the proliferation phase during angiogenesis. Integrins most expressed are  $\alpha v\beta 3$  and  $\alpha v\beta 5$ . Using these integrins as targets, will be therefore possible to obtain an antiangiogenic and anticancer effect.

There are several types of integrin inhibitors that have been evaluated in preclinical and in clinical trials: peptidomimetics (Cilengitide), antibodies (Intetumumab, Natalizumab), small organic molecules (E7820- MK0429)]. The advantage of small organic molecules compared to previous compounds lies in greater stability and in oral administration instead of intravenous.

Cilengitide (developed by Merck KGaA) is a new anti-angiogenic agent for the treatment of recurrent gliomas that act as an inhibitor of integrin receptors  $\alpha v\beta 3$  and  $\alpha v\beta 5$ , expressed on activated endothelial cells during angiogenesis. Integrins are proteins that facilitate the formation of new blood vessels within the tumor; blocking the receptor binding leads to the inability to invade the brain by the glioblastoma cells. This antitumoral and antiangiogenic effect of Cilengitide was demonstrated by several preclinical studies [68]. The molecule, in a phase II study presented at the American Society of Clinical Oncology (ASCO) in Chicago, was tested at two different doses (2 g or 500 mg twice a week) and provided very promising results in the group treated with the higher dose. Surprisingly, more than one third of patients in this group was still alive after one year and 22% after 2 years. 10% were still alive after 4 years [69].

Stupp et al. conducted a Phase I/IIa study of cilengitide and TMZ with concomitant radiotherapy followed by cilengitide and TMZ maintenance therapy in patients with newly diagnosed glioblastoma. Cilengitide at 500mg was administered as a 1-hour intravenous infusion twice weekly. The study highlighted how treatment with cilengitide, combined with the standard treatment, showed benefits in patients with MGMT promoter methylation. MGMT gene promoter methylation status was determined by methylation-specific polymerase chain reaction (PCR). The combination of cilengitide with TMZ and radiotherapy was well tolerated, without other side effects [70].

Merck is currently conducting two trials; the CORE study (cilengitide at a dose of 2 g twice weekly in combination with TMZ and radiotherapy) is a Phase II randomized, multicenter, open-label controlled trial investigating two regimens of intravenous cilengitide in combination with standard therapy (radiotherapy with concurrent and adjuvant TMZ) versus standard therapy alone, and is currently in progress in GBM patients with an unmethylated MGMT promoter.

The CENTRIC study is a multicenter, open-label, controlled phase III study, testing Cilengitide (at a dose of 2 g twice weekly) in combination with standard treatment (with TMZ and radiotherapy) versus standard treatment alone, in subjects with newly diagnosed glioblastoma multiforme and methylated MGMT gene promoter. Promising results in these trials probably will lead to a higher interest in integrins as targets for glioma therapy and to a bigger development of new anti-integrin agents. The results of clinical studies will be useful to explore the knowledge on the best dose of cilengitide used to obtain the desired therapeutic effect with lower side effects.

Recently the research of biomarkers predictive of patient response to anti-integrin agents has been directed toward the quantification of their expression through imaging, using radiolabeled galacto-RGD positron emission tomography, a new diagnostic technique [68].

## 12. Antisense approaches and mi-RNA inhibitors as anti-angiogenic agents

New antiangiogenic agents have been developed and tested in several preclinical studies with many results that encourage further research in this field. These are unconventional compounds such as siRNA and angiomiRs which differently act through gene silencing. This silencing mechanism has been used to interfere with the process of angiogenesis [61].

Other treatments to halt invasion involve genetic interventions using DNA, siRNA, and microRNA delivery via a host of transfection methods [71]. These therapies are all in animal phases at the moment, largely due to the invasive nature of the interventions and the difficulties in the past twenty years with gene therapy in clinical trials. The advantage of these interventions is the ability to directly target specific genes necessary for glioma invasion without need for design of a novel drug and determination of a particular target. However, like all compounds, there are delivery issues associated with these treatments as well as possible unforeseen side effects. Another kind of new possible antisense approach is represented by long non coding RNA, different RNA-based molecules than miRNA, probably transcribed not from intronic DNA sequences. All of these antisense approaches should be able to reach to knockout or down-regulate pathways pathologically involved in glioma progression and invasion, trying to reverse towards normality glioma cell genome or selectively kill only glioma cells. Into this fashionable therapeutic field, our study group is improving the study of possible involvement of IL-8 and HIF-1  $\alpha$  into high-grade glioma patients to hypothesize an antisense block of both these molecules at the same time with a single approach.

## 13. Other potential molecular targets

Recent discoveries in molecular biology have shown further potential signaling pathways involved in the pathogenesis of malignant gliomas. Were therefore assumed new targets for novel therapeutic approaches like growth factor ligands, receptors, intracellular downstream effectors. Several studies highlighted an over-expression of EGFR in malignant gliomas, which is related with survival and proliferation of cancer cells. EGFR tyrosine kinase inhibitors (TKIs) Gefitinib (Iressa®) and Erlotinib (Tarceva®), used in combination with ionizing radiation, can increase the antitumoral effect of this therapy. The results of several phase I/II studies using Gefitinib and Erlotinib with conventional therapy in the treatment of GBM are controversial [72]. For this matter were investigated other molecular markers (besides the over-expression of EGFR) more predictive of EGFR TKIs activity. Among these there are: EGFR variant III (EGFRvIII co-expressed when there is an amplification of EGFR), phosphatase and tensin homolog (PTEN) expression, and phospho-Akt (P-Akt).

Specifically, co-expression of EGFRvIII and the tumor-suppressor protein PTEN seems to be associated with a significant clinical response to EGFR TKIs but also these results are controversial. Further studies are needed to clarify the efficacy of these agents and to select patients who may benefit from EGFR inhibitor therapy. Imatinib (Glivec®), an antagonist of PDGFR (who may be overexpressed in high grade gliomas), showed in vitro on GBM cells cytostatic or cytotoxic activity in relation to its concentration [73]. This led to clinical trials in patient with recurrent glioma. A randomized, multicenter, open-label Phase III study of imatinib in combination with hydroxyurea versus hydroxyurea alone as oral therapy in patients with progressive pretreated GBM resistant to standard dose TMZ failed to show clinically significant differences between the two treatment arms [74].

Most experimental and more traditional anti-invasive therapeutic platforms have focused on the ability of compounds to inhibit matrix metalloproteinases (MMPs) and the other extracellular matrix (ECM) components and thus limiting invasive progress of cancer cells [75].

One such compound, Marimastat, an MMP-2 inhibitor, was developed and showed promise in some clinical trials, demonstrating a possible synergic effect when used with TMZ in anaplastic astrocytoma and recurrent GBM. Despite it, actually, it isn't sure a long term therapeutic effect better than TMZ alone [76, 77]. More recently, there has been a shift in the development of novel drugs to that of more personalized therapy. In these cases, specific receptors and signaling molecules are targeted for inhibition in order to kill cancer cells. The main goal to reach the selectivity and efficacy of antitumor treatment consist on targeted therapeutic molecular-based approaches. These therapies in the field of brain tumor and in particular in case of intra-axial brain tumor, such as high-grade gliomas, represent the most fascinating challenge to achieve the killing of glioma cells into the brain and, with an adequate engineering of targeted drugs and nanoparticles systems, the population of glioma and GBM stem cells. Treatment can be modified to the individual cancer type and patient which is advantageous in reducing side effects. They work often by relying on receptor addiction of the cancer cells meaning if the receptor becomes useless through blocking, the cancer cell will almost go through withdrawal and can die [78, 79]. However, this is not known to occur with all potential targets and it is always possible that pathways will be rerouted to compensate. This type of treatment often has a side effect of halting invasion of cancer cells though more commonly treatment leads to cell death. A recent review highlights many of these compounds and their potential anti-invasive effects based on the target molecule for which they were developed. However, the exact anti-invasive effects are unknown for most of these compounds based on lack of in depth studies of invasion opting instead for survival and tumor growth measures. Most of the targets of this type of therapy are receptor tyrosine kinases (RTKs) which, upon activation by a ligand, will change conformation and phosphorylate intracellular signaling proteins leading to downstream events. In cancer, these receptors are often mutated to be constitutively active and thus require no growth factors to function. A cancer cell can actually become "addicted" to this activation and so by turning it off, the cell will die. Inhibitors of RTKs are most often antibodies due to the high specificity. The more notable RTK inhibitors are Herceptin® (against EGFR), Avastin® (against VEGF), and Tarceva® (against PDGFR). These are all used clinically for different types of cancers and have been thought to have

possible anti-invasive effects due to their signaling pathways involving targets for invasion [80]. However, there is insufficient evidence to tie their effects to halting glioma invasion and as aforementioned, *in vivo*, Avastin causes glioma invasion. EGFR inhibition is particularly exciting due to the overexpression or mutation of EGFR in >60% of glioblastomas (de novo GBM) and alteration of glioblastoma to express EGFRvIII causes a more invasive phenotype of cells *in vitro* [81, 82]. However, these compounds are expensive and difficult to work with. Further, their actual role in invasion prevention is yet to be seen due to the far-reaching effects of the involved pathways.

Inhibitors of downstream proteins such as Src-kinase have shown promise in inhibiting tumor growth and progression. Based on the ability of Src to increase the invasive phenotype including cytoskeletal remodeling, MMP secretion, and adhesion disassembly, it was tested for anti-invasive activity. This treatment inhibited invasion in brain, prostate and breast cancers while also inhibiting growth, angiogenesis and proliferation [83].

Another new agent tested in clinical trials is Talampanel, a noncompetitive antagonist of the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. The receptor levels may be related with the GBM growth. This compound was tested in a multicenter Phase II study in association with standard therapy with RT and TMZ for newly diagnosed GBM. Patients showed a median survival of 20.3 months and 2 year overall survival of 41.7%. Only 29% of patients had MGMT promoter methylated [72].

## 14. Conclusions and future perspectives

Based on the results obtained from both the old and the most innovative therapies seems clear that a better understanding of the pathological and physiological mechanisms of brain cells can result in a more targeted therapeutic approach by designing personalized therapies. This would overcome the current limitations in the treatment and lead to a better prognosis for patients with brain tumors.

Nowday, the treatment of gliomas represents one of the most challenging areas in neurosurgery and oncology. Malignant gliomas involves multiple aberrant signaling pathways and the BBB restricts the delivery of many chemotherapeutic agents. The effectiveness of the actual chemotherapeutic approach, multimodal targeted therapies, differently than the other malignant extracerebral tumors, remains very modest in gliomas. Considering the multitude of molecular entities and signaling pathways regulating the proliferation and cellular survival/cell death, the inhibition of a singular target gene or transcriptional factor could not be sufficient to suppress neoplastic progression. In this intricate and complex field it seems to be very important to improve specific selective drug delivery systems to lead to diffusion of drugs, antisense oligonucleotides, small interference RNAs, engineered monoclonal antibodies and other therapeutic molecules into CNS overcoming BBB.

Considering the multitude of actually used chemotherapeutic approaches, starting from Stupp protocol (TMZ plus radiotherapy) and the results in term of recurrences and survival, the field



of glioma therapy is again subject to high rate of investigation and development with the hope to postulate new possible molecular targets and therapeutic schemes to attack glioma cells. Understanding the genetic bases of gliomas and of the invasive behaviour, in particular the differentiated gene expression in distinct areas of the same tumor during glioma progression, may suggest new molecular targets to overcome the mechanisms of multi-drug-resistance of the actual therapeutic approaches to gliomas and to attack at the same time different crucial biological events of gliomagenesis.

Glioma gene expression and its development during gliomagenesis will may help to better understand the role of important molecules involved in tumor-safe brain parenchyma relationships. These molecules, such as ECM proteases, cell adhesion molecules and their related signaling pathways show an important role in glioma cell migration and invasion and could be selectively downregulated to inhibit the glioma invasive rim. It is clear that the complexity and cross-talk between signal transduction pathways limits the potential efficacy of targeting a single receptor or molecule.

We are now investigating the role of HIF (Hypoxic inducing factor) isoforms and IL-8 (Interleukine-8) in glioma progression and the possibility to block their pathways through a only antisense molecule loaded into a nanoparticle-base system, and directed against both these molecular targets. HIF-1 $\alpha$  actively regulates downstream processes and it is also influenced by the tumor microenvironment in many different ways. Antisense inhibition of HIF may be a strong target for anti-angiogenic therapy. In fact, HIF is the crucial molecule and transcriptional factor produced in response to hypoxic conditions within HIF-1 $\alpha$ /VEGF-regulation expression-dependent angiogenic pathway. In this molecular cascade HIF-1 $\alpha$  up-regulated levels finally induce up-regulation of VEGF expression, and, at the same time, may stimulate gene expression of other genes involved in gliomagenesis.

Our group has recently demonstrated high expression levels of PGES-1 (Prostaglandine E 1 Synthase) and IL-8 in HGG cells and microglial cells, strongly correlated with grading tumor. During malignancy progression, leukocyte infiltration and necrosis are two biological phenomena associated with the development of neovascularization. IL-8 expression is first observed in low grade astrocytoma in perivascular tumor areas expressing inflammatory cytokines. In HGGs, IL-8 further localizes in oxygen-deprived cells surrounding necrosis. Macrophages are known to produce high levels of IL-8, which has a tumorigenic activity, by inducing tumor growth and angiogenesis; IL-8 is an inflammatory chemoattractant responding to the tumor microenvironment. Tumor pseudopalisading cells secrete hypoxic inducing factor (HIF) which induces IL-8 secretion. On the base of our preliminary results, we hypothesise an important role of IL-8 as crucial angiogenesis mediator within HIF-1 $\alpha$  pathway and crosstalk between hypoxia-induced high levels of HIF-1 $\alpha$  and VEGF expression. A simultaneous selective antisense strategy against two molecular targets involved at different levels in the same pathological pathway could be an attractive therapeutic mechanism aimed at enhancing an anti-angiogenic and anti-tumoral effect.

A very interesting new field of research and therapeutic application in glioma treatment is represented by studying and knowledge of differential phenotypic transcriptional profiles expressed into gliomas, and GBM in particular. Hoelzinger et al. in their study have showed

two different gene expression profiles into two distinct subpopulations of glioma cells, respectively the glioma cells located inside the stationary and proliferative tumor core and the glioma cells, located inside the motile and invading tumor rim. The authors report two different pattern of gene expression, characterized respectively by high levels of IGFBP2 and vimentin into tumor core glioma cells and upregulated expression of genes involved in adhesion, extracellular signal transduction, cytoskeletal rearrangement, intracellular signal transduction and apoptosis. In particular genes upregulated in invasive cells are autotoxin (ATX), the autocrin motility factor, protein tyrosine kinase 2 beta (PYK2), ephrin B3, antiapoptotic factor B-cell lymphoma-w (BCLW) and death-associated protein 3 (DAP3). The existence of two different gene expression profiles into distinct areas of the same tumor suggests the possibility to attack different molecular pathways and steps of glioma progression at the same time, targeting selectively different subsets of glioma cells. This introduces the possibility of multiagent treatment modalities, specifically targeting invasive cells, through new molecular therapeutic approaches in conjunction with classic treatments [84].

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