THE HETEROGENEITY AND THERAPEUTIC RESISTANCE OF GLIOBLASTOMA

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1. Abstract—Over the past decades, there has been an increasing use of molecular biology and genetic approaches in the assessment and management of adult gliomas, overall improving our understanding of the disease biology and behavior. Despite of these advances, maximal therapeutic intervention, as well as numerous new drugs entering clinical trials, the prognosis of malignant gliomas remains dismal. In this review, we provide an overview of the recent advances in the classification system, major molecular aberrations relevant to the biology of malignant gliomas, and standard as well as experimental treatment strategies. In addition, we discuss the complexity of this deadly disease, emphasizing the challenges associated with the enormously high degree of heterogeneity including cellular hierarchies at the inter- and intra-patient level.

2. Introduction

Malignant tumors of the brain are a rare occurrence accounting for approximately 2% of all cancers in adults. The term 'brain tumor' refers to a mixed group of neoplasms originating from intracranial tissues and the meninges with degrees of malignancy ranging from benign to aggressive (McKinney, 2004; Westphal and Lamszus, 2011). Individual brain tumors differ by their unique biology, treatment, and prognosis and each is likely to be caused by different risk factors. Due to their location in eloquent brain areas, even 'benign' tumors can be lethal. The survival of brain tumor patients is strongly correlated with the age, histologic subtype and degree of malignancy, as well as presenting symptoms. Gliomas are the most frequent primary brain tumors (approximately 70%) in adults of unknown cause and origin. The yearly incidence is six cases per 100 000 (Ricard et al., 2012). Even though the cause of gliomas remains elusive, the exposure to ionizing radiation is a known risk factor (Crocetti et al., 2012). A genetic predisposition to gliomas is well known in the setting of rare familial tumor syndromes (among others: type 1 and type 2 neurofibromatosis as a result of NF1 and NF2 mutations; LiFraumeni syndrome due to TP53 mutations, Cowden syndrome due to PTEN mutations). The genetic contribution to familial glioma is not well understood. Using whole exome sequencing of 90 individuals from 55 families, Bainbridge et al. (2014) identified two families with mutations in POT1 (p.G95C, p.E450X), a member of the telomere shelterin complex, shared by both affected individuals in each family and predicted to impact DNA binding and TPP1 binding, respectively (Bainbridge et al., 2015).

Altogether with the notoriously known radio- and chemo-resistance of malignant gliomas prompts the collective need for a further understanding of the molecular mechanisms underlying GBM development in relation to therapeutic strategies, which will be the focus of this review.

3. Classification

3.1. Histo-pathological classification

Gliomas are classified into three distinct categories based on tumor cell morphology and similarities between tumor and mature normal glial cells: astrocytomas, oligodendrogliomas and mixed oligoastrocytomas, the largest group being represented by astrocytomas (approximately 80%) (Westphal and Lamszus, 2011). To predict tumor behavior and patient survival, astrocytomas are further classified according to the World Health Organization (WHO) classification system based on histo-pathological criteria into four grades (WHO grade I-IV) (Louis et al., 2007). The WHO grade I tumors, pilocytic astrocytomas, are biologically benign and are considered to be potentially curable by surgical resection alone. The WHO grade II, diffuse astrocytomas, tend to infiltrate the surrounding parenchyma complicating complete surgical resection; WHO grade III, anaplastic astrocytomas, are characterized by increased cell density, anaplasia and proliferation. Lastly, WHO grade IV, Glioblastoma multiforme (GBM), differs from the other grades with the display of microvascular proliferation, necrosis and widespread infiltration of surrounding parenchyma (Louis et al., 2007; Ricard et al., 2012). GBMs are further divided based on their pathological development into two distinct groups, namely primary and secondary GBM. The more common primary GBMs present themselves *de novo* with no

prior history of lesions. They are characterized by chromosome 9p and 10q losses as well as amplification or alteration in expression of the Epidermal Growth Factor Receptor (EGFR) (Huse et al., 2013; Talasila et al., 2013; Verhaak et al., 2010). Secondary GBMs develop via a progressive pathway from pre-existing lower grade astrocytomas. Tumors developing through this pathway have distinctive genetic and clinical characteristics, and are associated with a better prognosis (Kim et al., 2010). On a molecular level, secondary GBMs are characterized by mutations in Isocitrate Dehydrogenase 1/2 (IDH1/2), which have been proposed to be initiating events in glioma development (Juratli et al., 2012a; Juratli et al., 2012b; Parsons et al., 2008).

GBM is the most common and malignant form of brain tumor and although cancers of the nervous system only represent <2% of all cancers, GBM lethality ranks among the highest (Olar and Aldape, 2014; Westphal and Lamszus, 2011). Since the 1970s, maximal safe resection combined with radiation therapy has been the mainstay of GBM treatment. Chemotherapy was a debated subject until 2005, where temozolomide (TMZ) in combination with radiation therapy, was shown to increase the median survival of patients with newly diagnosed GBM from 12.1 to 14.6 months (Stupp et al., 2005b; Stupp and Roila, 2009). Despite maximal safe resection followed by radiotherapy in combination with TMZ (the standard of care) and various salvage therapies at recurrence, the majority of patient succumbs to this disease within 2 years of diagnosis (Tanaka et al., 2013), and so the prognosis for GBM patients remains dismal. Following TMZ treatment after GBM recurrence, only 21% of patients obtain a progression-free survival (PFS) of six months and a six month overall survival (OS) of 60% (Yung, 2000; Yung et al., 2000). Despite concerted efforts, the prognosis is poor with a 5-year mortality rate of more than 90%, a number that has not improved for the last 30 years (Carlsson et al., 2014). The median survival rate of just 14.6 months after initial diagnosis is accredited the unique limitations such as tumor accessibility, intra- and inter-patient heterogeneity, infiltrative capacity as well as a poor pathophysiological understanding (Carlsson et al., 2014; Stupp and Roila, 2009).

The understanding of gliomagenesis has over the last years changed radically with the development of classification and categorization of molecular markers. The major weakness of the traditional WHO classification is its lack of reproducibility, subjective character of criteria used, and most importantly it remains imperfect in its ability to predict individual outcome (Ricard et al., 2012). Thus, major effort has been made in order to identify and validate clinically relevant prognostic and predictive biomarkers. Recently implemented markers in the gliomas diagnostics include 1p/19q co-deletion, methylation of the O-6 methyl-guanine-DNA methyltransferase (MGMT) gene promoter, alterations in the epidermal growth factor receptor (EGFR) pathway, and isocitrate dehydrogenase 1 (IDH1) gene mutations (Agnihotri et al., 2014; Lai et al., 2011; Verhaak et al., 2010) *3.2. Molecular classification*

As ‘multiforme’ denotes, GBM exhibits distinct inter- and intra-patient heterogeneity with cellular hierarchies and cancer stem cells at their apex. Although morphologically identical, different GBM tumors translate into different clinical outcomes. With the increased pace of high-throughput genomic technologies and large scale profiling efforts, a seminal study was published by Phillips et al. (Phillips et al., 2006), who used unsupervised hierarchical clustering of expression profiles and characterized 3 GBM subtypes: proneural, proliferative and mesenchymal. In 2005, The Cancer Genome

Atlas (TCGA) was established by the US National Cancer Institute and National Human Genome Research Institute and initiated systematic and comprehensive analysis of gene copy number, mRNA expression, and the epigenetic state of untreated primary GBMs (2008). The proneural subtype was associated with earlier onset, platelet-derived growth factor receptor A (PDGFRA) aberrations, IDH1 and TP53 mutations. A study by Vitucci et al. (2011) suggested that the proneural subtype generally correlates with marginally improved survival, whoever being resistant to TMZ and radiation therapy (Vitucci et al., 2011). The mesenchymal subtype is typical by high expression of CHI3L and genomic loss of NF1, TP53 and PTEN usually showing a poor outcome compared to other subtypes. The classical subtype exhibits EGFR amplifications/vIII mutations without IDH1 and TP53 mutations, but frequent PTEN loss. (For a comprehensive overview of GBM origin and classification, see **Figure 1**).

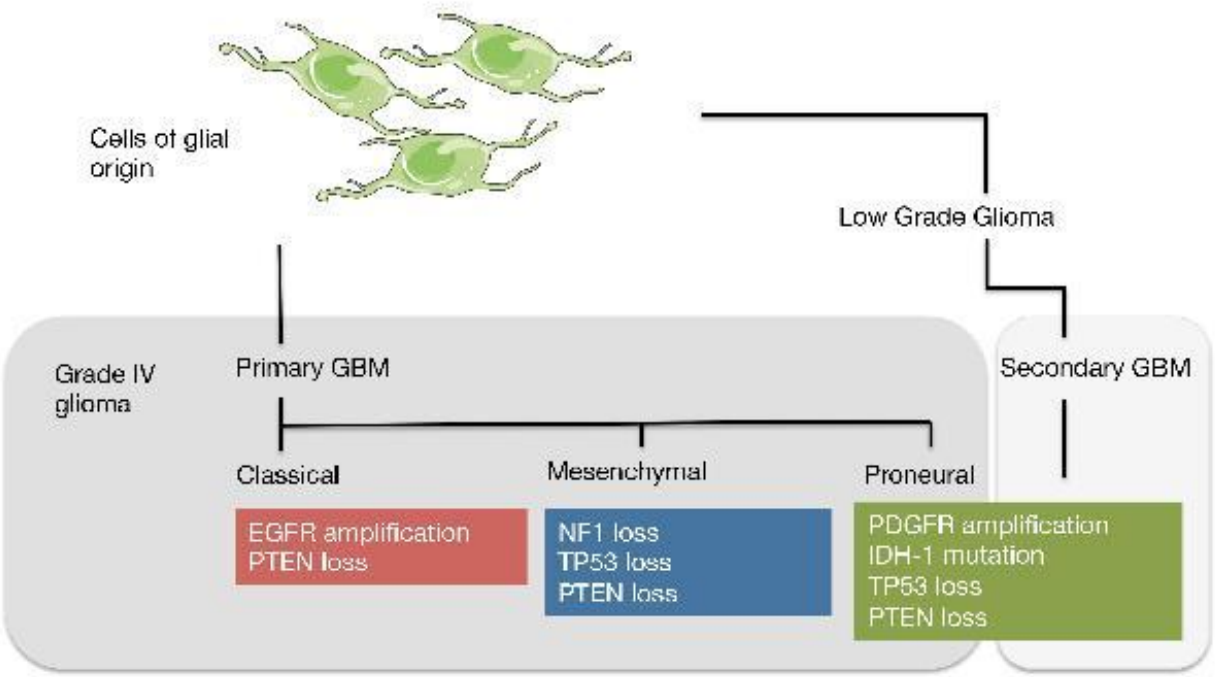

Figure 1. GBM origin and classification. GBM is proposed to originate from cells of glial morphology and it can occur *de novo* in a fully malignant state (primary GBM) or it may develop via progressive pathway from lower grades of malignancy (secondary GBM). With recent developments of sequencing technologies, GBM tumors, which are well-known by their heterogeneity have been classified into molecular subtypes, the major 3 being: classical (red); mesenchymal (blue) and proneural (green). The most common types of genomic alternations associated with individual subtypes are listed in boxes below. For more details, see text.

The study by Sottoriva et al. (2013) revealed the genome-wide architecture of intra-tumoral variability in GBM across multiple spatial scales, thereby revealing the tumor evolution. They reported that based upon gene expression levels, tumor fragments from the same patient may be classified into different subtypes (Sottoriva et al., 2013). Whether the expectations of the molecular classification will hold promises and have any prognostic and/or predictive significance remains to be determined by future studies.

4. Cancer Stem Cell

4.1. Cancer Stem Cells hypothesis

One of the proposed causes of the high recurrence rates in GBM is the repopulation by treatment resistant cells. These so called 'Cancer Stem Cells (CSCs)' are believed to have unlimited proliferation potential, self-renew and produce multi-lineage differentiated progeny (Yan et al., 2013). The proposal that diseases arise from activation of stem-like cells was introduced more than a 150 years ago, when the German 'father of pathology' Rudolf Virchow presented "o*mnis cellula e cellula*" ("every cell stems from another cell") (Rahman et al., 2011). Virchow proposed that cancer does not simply appear spontaneously, but that it arises from the activation of dormant remnants of embryonic tissue. In 1997, CSCs were described in leukemia and later confirmed in a range of solid tumors, including GBM (Barraud et al., 2007; Hemmati et al., 2003; Morrison and Spradling, 2008; Patrawala et al., 2007; Sussman et al., 2007; Uchida et al., 2000). The discovery of CSCs prompted the formulation of the stem cell or hierarchical model, in which a subset of rare cells gives rise to multi-differentiated progeny, maintains tumor growth and recapitulates patient's phenotype in immunocompromized hosts (Shackleton et al., 2009). The conceptualization of this model has challenged the original stochastic or clonal model for tumor evolution, in which any individual cell has equal potential to transform into cancerous cell with unlimited proliferative capacity. These two models do not contradict each other, they are rather complementary and add further complexity to GBM evolution; moreover, altogether may form a basis for development of more effective therapies (**Figure 2**).

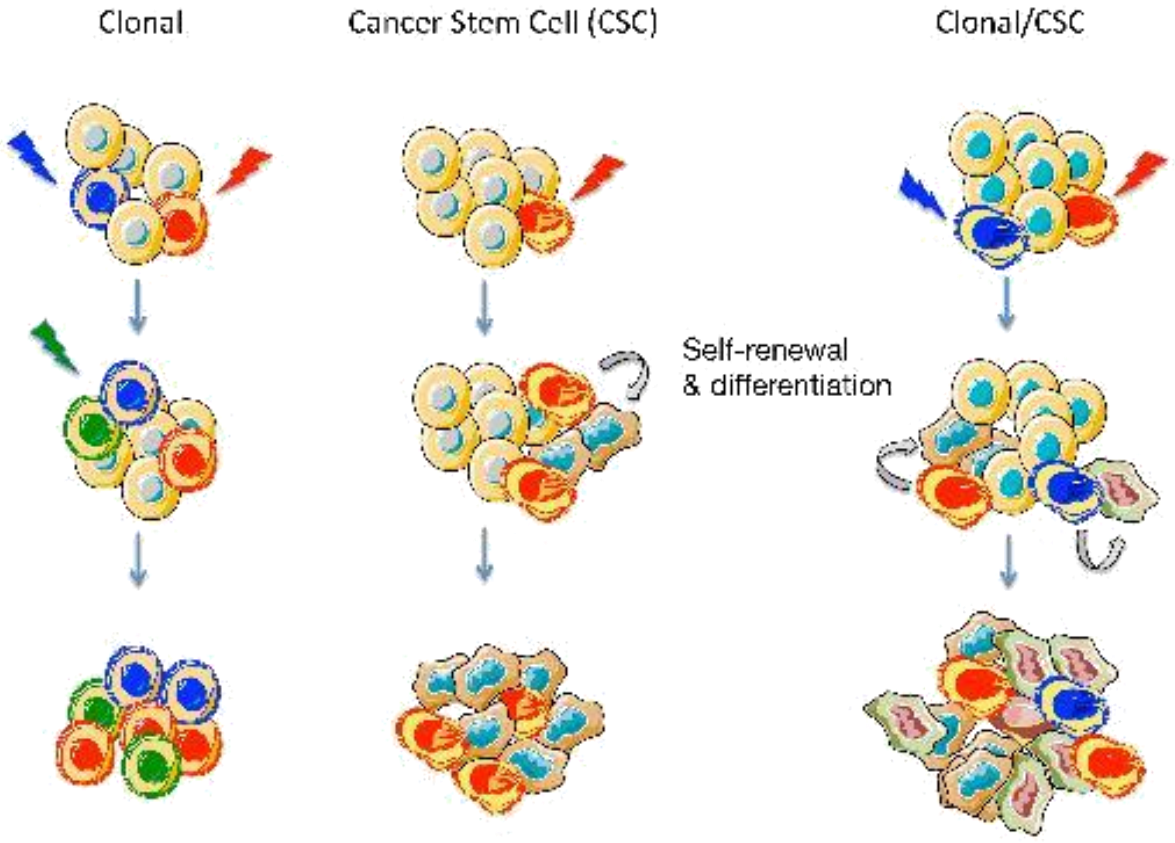

Figure 2. A. The stochastic model of tumorigenesis suggests that any tumor cell has the same predisposition for malignant transformation and that tumors are composed of clonal populations carrying various genomic aberrations. **B**. On the other hand, the hierarchical or CSC model suggests that there is a rare subset of cells, so-called CSCs, that is capable of self-renewal, differentiation and tumor propagation/maintenance. **C**. The two models (A and B) are not mutually exclusive, but they rather complement each other. There are multiple 'clonal' CSC populations capable of self-renewal and tumor maintenance. (The graphics of individual cells was adopted from Servier Medical Art)

4.2. Cancer Stem Cell Identification

Up to date, the most commonly used marker for isolation and identification of CSCs in GBM is cell surface glycoprotein CD133. CD133, also known as Prominin-1, was initially discovered as a cell surface marker for hematopoietic stem cells (Miraglia et al., 1997). In 2003, Singh et al. showed that orthotopic injection of as few as one hundred cells positive for CD133 into immunocompromised mice was sufficient to initiate tumor growth, whereas as many as one hundred thousand cells negative for CD133 could not (Singh et al., 2003; Singh et al., 2004). The functional role of CD133 is largely unknown, although it has recently been demonstrated to be involved in pro-survival signaling upstream from the PI3K/AKT pathway (Fargeas et al., 2003; Wei et al., 2013).

In the light of molecular GBM sub-classification, investigations identified the presence of subtype-specific CSC pools (Bhat et al., 2013; Mao et al., 2013). The authors have shown that several other biomarkers can be used to isolate CSCs. One of these, CD44 (also referred to as Home Cell Adhesion Molecule (HCAM)), is a cell surface molecule expressed in multiple tumors as well as in normal tissue where it regulates cell migration, proliferation and survival (Jijiwa et al., 2011). Furthermore, CD44 has proposed as a complementary marker to CD133 for CSC isolation of the mesenchymal subtype (Bhat et al., 2013). Numerous other markers have been suggested for CSC identification including CD15, A2B5, ALDH1, Olig2, Nanog, Oct4 and Mushashi-1(Cheng et al.,

2011; Douville et al., 2009; Kim et al., 2013; Patrawala et al., 2005; Son et al., 2009; Wang et al., 2008b; Zhu et al., 2012). Although several techniques used to isolate CSCs from tumor bulk have been developed, debates regarding the consistency and lack of universal markers remain (Bidlingmaier et al., 2008; Broadley et al., 2011; Wang et al., 2008a).

4.3. Cancer Stem Cells and Therapeutic Resistance

4. 3. 1. Anti-angiogenic therapy resistance

Preclinical studies show that GBM-derived CSCs display elevated VEGF expression, form highly angiogenic tumors in immunocompromised mice and reside in perivascular niches in close contact with endothelial cells (Calabrese et al., 2007). Traditionally, VEGFR2 expression has been attributed endothelial cells, but recently, there has been evidence of increased VEGFR2 expression in GBM-derived CSCs (i.e. GSCs) (Hamerlik et al., 2012). Moreover, the work by Wang et al. (2010) and Ricci-Vitiani et al. (2010) has proposed that the GSCs have the potential to trans-differentiate into endothelial progenitors and so contribute to vessel formation. These findings were supplemented by Cheng et al. (2013), who has provided *in vivo* evidence of GSCs' differentiation into pericytes (Cheng et al., 2013). The functional paracrine as well as autocrine VEGF/VEGFR2 signaling, and ability to contribute to angiogenesis via differentiation into functional endothelia and pericytes, gives GSCs predisposition to be a source of GBM's resistance to anti-angiogenic therapies.

4. 3. 2. Resistance to DNA damaging therapies

It has been demonstrated that after ionizing radiation (IR), there is an increase in the GSCs population both *in vitro* and *in vivo*. The preferential survival of GSCs (in comparison to their differentiated counterparts) is mainly due to lower rates of apoptosis and constitutive activation of DNA damage response pathways (DDR) leading to higher DNA repair capacities of these cells (Bao et al., 2006). After radiation, the DDR are activated and cells arrest in cell cycle in order to repair DNA breaks. The activation of DDR in GSCs occurs through the cell surface adhesion protein and GSC marker, L1CAM (CD171) (Cheng et al., 2011). L1CAM together with other proteins involved in DDR (ATM, Rad17, Chk1 and Chk2) are therefore 'hot' candidates for GSCs' sensitization to IR. Another putative therapeutic target is the polycomb group protein, BMI1. It has been suggested that BMI1 is enriched in CD133 positive cells and is essential for GCSs ability to self-renew independently of the INK4A/ARF-pathway and mainly through the transcriptional suppression of alternate tumor suppressor pathways (Facchino et al., 2010). Following radiation, BMI1 is redistributed to the chromatin, where it co-localizes with several proteins that are necessary in the DNA double-stranded break (DSB) repair (Facchino et al., 2010). Apart from DNA damage checkpoint response a number of other pathways contribute to radioresistance including autophagy and stem cell maintenance pathways (Bar, 2011; Beier et al., 2011; Hambardzumyan et al., 2008; Venere et al., 2013b; Yan et al., 2013). The stem cell maintenance pathways such the Notch, PI3/Akt and Wnt/b-catenin signaling are also suggested to be driving the radio-resistance of GSCs. The Notch pathway has been shown to promote radioresistance in GSCs through activation of the PI3K/Akt pathway and the up-regulation of the pro-survival protein, Mcl-1(Wang et al., 2010). The PI3-akt pathway also is suggested to have a role in radio-resistance upon the activation of the insulin-like-growth factor (IGF-1) receptor and secretion of IGF1 from GCSs. Treatment with IGF-1 receptor inhibitors has shown to increase radio-sensitivity (Osuka et al., 2013).

The existence of CSCs is a fairly novel concept in cancer research and their biology as well as resistance to conventional therapies must be further explored (**Figure 3**). With the development of methods for CSC identification and isolation, the studies of their biology and behavior will advance; only then CSC-directed therapies might become useful therapeutic strategy for GBM.

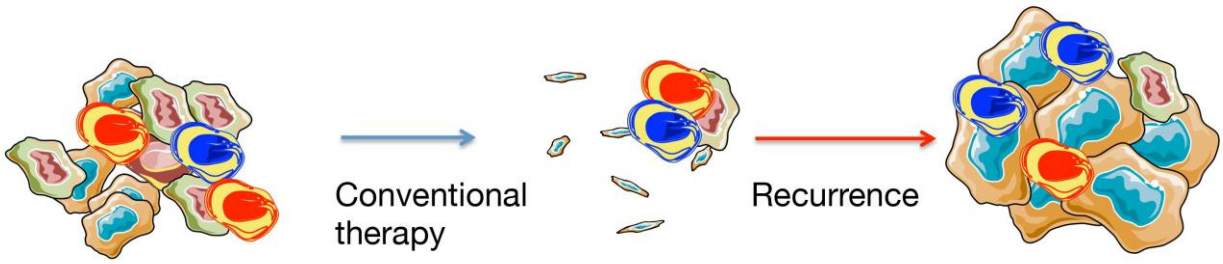

Figure 3. CSCs contribute to therapeutic resistance of GBM. Conventional therapy has been developed to target the tumor bulk, neglecting the radio- and chemo-resistant CSCs. After conventional therapy, CSCs remain serving as a repository for tumor relapse, often in a more aggressive manner. (The graphics of individual cells was adopted from Servier Medical Art)

5. Therapeutic intervention

5. 1. Anti-angiogenic therapies

It has been a common idea since Judah Folkmans discoveries in 1971 that, as with normal tissue, cancerous tissue needs a steady supply of nutrients in order to propagate and survive (Folkman, 1971). GBM is among the most vascularized tumors, where angiogenesis is largely driven by Vascular Endothelial Growth Factor / Vascular Endothelial Growth Factor Receptor (VEGF/VEGFR) signaling. In mammals 5 different VEGFs have been identified (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PLGF)), VEGF-A is a key factor in tumor angiogenesis (Holmes et al., 2007) and its expression was found to be elevated in many solid cancers, among those GBM (Berse et al., 1992).

Due to higher degree of vascularization, targeting angiogenesis through VEGFR-VEGFR2 signaling has been a major focus in recent drug development. Bevacizumab is a humanized monoclonal antibody against VEGF-A and was originally approved by the US Food and Drug Administration (FDA) for the treatment of metastatic colorectal cancer in combination with chemotherapy (Hurwitz et al., 2004). In 2009, the FDA approved bevacizumab for the treatment of recurrent GBM on the basis of radiographic response rates ranging from 28-59% (Cohen et al., 2009). Despite initial optimism and much ensuing research, there are many uncertainties and unanswered questions in relation to the often-observed resistance accompanied by a metabolic switch, invasive and much more aggressive phenotype (Bloch et al., 2013; Carbonell et al., 2013; DeLay et al., 2012; Jahangiri et al., 2013). Bevacizumab has also been explored in randomized phase II trials in combination with standard chemoradiation in patients with newly diagnosed GBM, including the Radiation Therapy Oncology Group (RTOG) 0825 study in the USA and AVAglio in Europe and Asia. In the Avaglio-study, bevacizumab improved median PFS from 6.2 to 10.6 months, and similar effects were reported by the RTOG-study (PFS improvement from 7.3 to 10.7), although this did not meet the predefined PFS significance level (Chinot et al., 2014; Gilbert et al., 2014). Despite reservations and disappointment in the neuro-oncology community, bevacizumab remains the only targeted therapy objectively exhibiting clinical efficacy compared with

chemotherapy alone among patients with recurrent disease. The ability to identify predictive biomarkers and select those patients, who are most likely to benefit, would be ideal.

5. 2. Molecular-targeted therapies

The highly aggressive behavior of GBM tumors is not only due to genetic abnormalities but also aberrant signaling. Among many key signaling pathways altered in GBM are the receptor tyrosine kinase (RTK)/phosphatidylinositide-3-kinase PI3K/Akt, TP53/MDM2, RB/cycling-dependent kinase 2A (CDKN2A), Ras/extracellular signal-regulated kinase (ERK), and transforming growth factor (TGF) (Nakada et al., 2011; Tanaka et al., 2013). Furthermore, protein kinase C (PKC) pathways and Janus kinase (JAK) signal transducer and transcription activators such as STAT are also associated with gliomas (da Rocha et al., 2002; Silva, 2004). The rationale of using targeted molecular therapies is that this approach is highly specific towards tumor cells potentially resulting in less toxic side effects than with the use of chemotherapy (Chi and Wen, 2007). A number of therapeutic approaches have been considered in order to disturb protein kinase signaling including small-molecule inhibitors, monoclonal antibodies (mAbs), antisense oligonucleotides, conjugation of ligand toxins to receptor kinases and ribozymes (Carlsson et al., 2014; Huse et al., 2013).

5. 2. 3 EGFR receptor signaling

EGFR signaling in normal healthy cells is essential during embryogenesis and glial development and is believed to be the upstream regulator of two of the most critical pathways driving gliomagenesis: Ras-MAPK pathway and PI3K-Akt pathway (Westphal and Lamszus, 2011). In GBM, over-expression of EGFR and/or its constitutively activated variant EGFRvIII is a major characteristic and is associated with tumorigenesis and more aggressive phenotypes, such as, invasiveness and therapeutic resistance. The clinical efficacy of EGFR-targeted therapy has been only modest in GBM patients. Although intrinsic drug resistance is known to be a major obstacle for EGFR-targeted therapy, the underlying mechanisms are still poorly understood, despite extensive investigations are being conducted to shed light on these mechanisms (Lo, 2010a; Lo, 2010b).

5. 2. 5. 2. 4. PDGFR receptor signaling

PDGF and their receptors PDGFRs also play an important role in the regulations of embryogenesis and glial development in normal cells (Richardson et al., 1988). The contribution of PDGF signaling toward glioma formation in mice has been well documented. Injection of a PDGF-B chain-encoding retrovirus into the brain of newborn C57B16 mice induced brain tumor formation in 40% of animals (Uhrbom et al., 1998). Imatinib mesylate (Gleevec; formerly known as STI571) is a potent inhibitor of the Bcr-Abl,α **PDGFR**β,c -Fms, and c-Kit tyrosine kinases. Its ability to inhibit PDGFR with an IC_{50} of 0.1 μmol/L suggested that it might have therapeutic potential in malignant gliomas. Kilic et al. found that imatinib inhibited the growth of U343 and U87 glioblastoma cell lines *in vitro* and *in vivo* at concentrations achievable in man, providing support for its potential therapeutic value in patients with malignant gliomas (Kilic et al., 2000). However, single-agent imatinib seems to have only minimal activity in malignant gliomas and may be associated with a slightly increased risk of intratumoral hemorrhage (Wen et al., 2006). The mechanism underlying the hemorrhage is unclear, but suggested to be associated with the PDFGR signaling, while the low therapeutic efficacy was ascribed to a limited penetration of the drug through the blood brain barrier (BBB). This has lead to the engineering of second-generation PDGFR inhibitors that have

improved BBB penetration MLN-518 and dasatinib. Several clinical trials are currently testing the efficacy of dasatinib in combination with chemotherapy or radiation in a number of different gliomas. Recently, a phase III clinical trial investigated the effect of combination therapy with MLN-518 and bevacizumab (www.clinicaltrials.gov). The study provided evidence that MLN-518 induces reversible muscle weakness and electrophysiological changes consistent with neuromuscular junction dysfunction in patients with recurrent gliomas (Lehky et al., 2011). Thus, future results from ongoing trials may determine the efficacy and tolerability of PDGFR inhibition in gliomas.

5. 2. 5. Miscellaneous pro-survival signaling

Apart from the abovementioned strategies, a number of molecular targeted therapies aimed at inhibition of other regulators of cell survival and proliferation are undergoing clinical trials (www.clinicaltrials.gov). These include novel targets such as proteasome, matrix metalloproteinases, adhesion molecules and chromatin remodeling factors (Wang et al., 2015). The efficacy and success of molecular targeted therapies has been limited by diverse factors, ranging from complexity of molecular biology underlying gliomagenesis to challenges of patient selection to specific therapies (above mentioned heterogeneity, drug delivery, and evaluation of treatment response). Better understanding of what role these factors play might indicate the appropriate direction for development of molecular targeted therapy in malignant gliomas.

5. 3. DNA damaging therapies

Cancer chemotherapeutic agents and radiotherapy exert their cytotixc effects by inducing DNA damage. The constitute and maximal activation of DDR already in low grade gliomas (Bartkova et al., 2010) and recent reports that loss of key DDR factors accelerates tumor formation in mouse models (Squatrito et al., 2010) underlines the importance of DNA repair mechanisms as a significant contributor to therapeutic resistance and identifies a number of novel druggable targets to be validated in clinical trials. In the last two decades, a number of alkylating agents (namely nitrosoureas, ACNU-nimustine; BCNU-carmustine, and CCNU-lomustine) have been used with more or less success in the treatment of gliomas (Walker et al., 1978; Weller et al., 2003). Nitrosoureas, mainly CCNU's, induce more prolonged G2-M arrest resulted in a much higher number of cells undergoing apoptosis (Hirose et al., 2001). CCNU's alkylate DNA (guanine at the N7-position and adenine at the N3-position) (Batista et al., 2007; Fischhaber et al., 1999) and chloroethylates DNA (O6-position of guanine, leading to N1 - deoxyguanosinyl-N3-deoxycytidyl cross-link) resulting in DNA strand breaks during mitosis and thus cytotoxity. Other than influencing DNA-repair system, CCNUs are also believed to induce cell death through p53 status (McCord et al., 2009).

The alkylating agent TMZ is administered to GBM patients concurrently with radiotherapy and has been suggested to instigate tumor cell death through mainly through DNA damage (Yung, 2000). In the first cell cycle post treatment, TMZ methylates the O6-position of guanine, the primary lesion, which results in mismatch with thymine in double-stranded DNA (O6G-T) and the recurrent GT-mismatches resulting in cycles of mismatch repair are then essentially futile. These mismatch repair cycles leads to double strand breaks or recombinogenic lesions, the secondary lesion. (Kaina et al., 1997; Karran and Bignami, 1994; Karran et al., 2003; Roos and Kaina, 2006). Apart from DNA damage, TMZ has been suggested to induce temporary G2-M arrest mediated through p53 and p21WAF1/Cip1 in TP53 deficient cells contributing to tumor death. The administration of TMZ in combination with

maximal-safe surgical resection and radiotherapy in GBM has been successful (as it resulted in a significantly improved OS/ PFS accompanied with low levels of toxicity) and therefore became a standard of care (Beier et al., 2009; Hau et al., 2007). However, the proportion of long-term survivors is still very low (Stupp et al., 2005a). A number of causes to tumor recurrence (after maximal radio-chemotherapy) has been suggested including, poor drug delivery and chemoresistance. CSCs are believed to be the mediators of this resistance, as they are able to survive chemotherapy and assumingly later initiate tumor recurrence in accordance with the cancer stem-cell hypothesis. CSCs survive chemo-therapy because of a number of intrinsic factors including the MGMT methylation, disturbed mismatch repair system, Poly (ADP-ribose) polymerase (PARP-1) hyperactivation, de-regulation of apoptosis-regulating genes (EGFR, MDM2, and Bcl-2) and molecular transporter proteins overexpression to actively efflux chemotherapeutic agents out of the tumor cells (Sarkaria et al., 2008; Venere et al., 2013a). Based on the successful *in vitro* studies, various PARP inhibitors are in Phase I and II clinical trials (for examples ABT-888, CEP-6800, AG014699, GPI15427) (Powell et al., 2010). In the clinic, MGMT is used as a predictor of therapeutic response to alkylating agents; results from on going trials will have to elaborate on the correlation between MGMT and response to chemotherapy (www.clinicaltrials.gov). Additionally, PARP-inhibitors are also being considered to overcome chemo-resistance, in fact a number of anticipated clinical trials on-going, planned and completed will evaluate the effect of PARP-inhibitors in both newly diagnosed and recurrent gliomas in combination therapy with other alkylating agents (www.clinicaltrials.gov).

6. Concluding remarks

In this review, we have summarized and discussed current understanding of the biology and therapeutic resistance of GBM, which is among the deadliest of solids cancers. GBM is a heterogeneous disease encompassing a very complex morphology and physiology, albeit contributing to its renown therapeutic resistance. Despite recent advances of GBM's molecular biology, the prognosis for GBM is poor and no alternative treatment options exist. **Tables 1-3** show selected anti-angiogenic and molecular targeted therapies of malignant gliomas, which are already in clinical trials. With the emerging molecular classification and deeper understanding of molecular pathways associated with GBM therapeutic resistance, new prognostic and predictive biomarkers may lead to more personalized approach. It is very likely, that genotypic analysis will become part of standard of clinical care as a surrogate to histopathologic evaluations. However, it is currently unknown whether molecular classification yields a more accurate risk prediction and so improve significantly patient outcome when compared to current standard of care. The first step towards more efficient treatment development, of maximal benefit and minimal toxicity, a thorough understanding of the molecular as well as cellular plasticity in the context of cellular hierarchies is essential.

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Table 1: Selected anti-angiogenic drugs in newly diagnosed or recurrent glioma studies. Clinical trials listed in this table are registered with active status (open, recruiting or ongoing) or completed as of 2014-2016.

Target	Molecular agent	NCT Number	Phase	Status	Tumor type	Overall survival	Progression free survival
VEGFR							
	PTC299	NCT01158300	Phase 1	Completed. Study completion: January 2015	Refractory or recurrent primary CNS tumor.	-	-
	Bevacizumab + Vorinostat	NCT01266031	Phase 1/2	Active, not recruiting. Primary Completion: July 2016	Newly diagnosed glioma.	-	-
	Bevacizumab + NKTR-102	NCT01663012	Phase 2	Active, not recruiting. Study completion: November 2015	Resistant high-grade glioma.	-	-
	Bevacizumab	NCT00271609	Phase 2	Completed. Study completion: February 2014	Recurrent high-grade glioma.	-	Anaplastic glioma: 20,9 % 6 month PFS. GBM: 29% 6 month PFS.
	Dovitinib	NCT01753713	Phase 2	Active, not recruiting. Study completion: December 2014	Recurrent or progressive glioblastoma.	-	-
	Bevacizumab + Temozolomide	NCT01149850	Phase 2	Recruiting. Primary completion: July 2015	Newly diagnosed glioblastoma.	-	-

Table 2: Selected receptor tyrosine kinase inhibitors in newly diagnosed or recurrent glioma studies. Clinical trials listed in this table are registered with active status (open, recruiting or ongoing) or completed as of 2014-2016.

Target	Molecular agent	NCT Number	Phase	Status	Tumor type	Overall survival	Progression free survival
EGFR							
	Erlotinib	NCT01257594	-	Active, not recruiting. Study completion: December 2014	Recurrent glioma.	-	-
	Afatinib (BIBW2992) + Radiation + Temozolomide	NCT00977431	Phase 1	Active, not recruiting. Study completion: June 2015	Newly diagnosed glioblastoma.	-	-
	Erlotinib + Radiation	NCT00124657	Phase 1/2	Completed. Study completion: September 2014			Anaplastic astrocytoma Phase 1: 0.75 years Phase 2: 0.45 years GBM: Phase 1: 0,33 years Phase 2: 0,19 years
PDGFR							
	Nilotinib	NCT01140568	Phase 2	Recruiting. Study completion: June 2016	Recurrent glioblastoma.	-	-
	Crenolanib	NCT01393912	Phase 1	Active, not recruiting. Primary completion: September 2014.	Recurrent high-grade glioma.	-	-

Table 3: Selected DNA-alkylating agents in newly diagnosed or recurrent glioma studies. Clinical trials listed in this table are registered with active status (open, recruiting or ongoing) as of 2015-2019. PARP-1 inhibitor: Poly (ADP-ribose) Polymerase-1 inhibitor. DNA-PK inhibitor: DNA-dependent protein kinase inhibitor.

Name	Type	NCT Number	Phase	Status	Tumor type
Temozolomide + Radiation	DNA alkylating agent	NCT00482677	Phase 3	Active, not recruiting. Study completion: July 2015	Newly diagnosed glioblastoma.
Temozolomide + Bevacizumab	DNA alkylating agent	NCT01149850	Phase 2	Recruiting. Primary completion: July 2015	Newly diagnosed glioblastoma.
Temozolomide + Folic acid	DNA alkylating agent	NCT01700569	Phase 1	Recruiting. Study completion: October 2014	Glioblastoma.
Temozolomide + Olaparib	DNA alkylating agent	NCT01390571	Phase 1	Recruiting. Primary completion: September 2015	Recurrent glioblastoma.
Temozolomide + Memantine + Mefloquine + Metformin	DNA alkylating agent	NCT01430351	Phase 1	Recruiting. Primary completion: September 2016	Glioblastoma.
BSI-201	PARP-1 inhibitor	NCT00687765	Phase 1/2	Active, not recruiting. Study completion: June 2015	Newly diagnosed high-grade glioma.
Olaparib + Temozolomide	PARP-1 inhibitor	NCT01390571	Phase 1	Recruiting. Primary completion: September 2015	Recurrent glioblastoma.
Veliparib + Temozolomide + Radiation	PARP-1 inhibitor	NCT01514201	Phase 1 /2	Recruiting. Primary completion: August 2019	Diffuse pontine gliomas.
CC-122	DNA-PK inhibitor	NCT01421524	Phase 1	Recruiting. Study completion: September 2016	Glioblastoma and other tumors outside of CNS.
MK-1775 + Radiation	WEE-1 kinase inhibitor	NCT01922076	Phase 1	Recruiting. Primary completion: August 2017	Newly diagnosed diffuse intrinsic pontine gliomas.
MK-1775 + Temozolomide + Radiation	WEE-1 kinase inhibitor	NCT01849146	Phase 1	Recruiting. Primary completion: June 2017	Newly diagnosed glioblastoma.

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