Gliomas

Waldemar Debinski, MD, PhD
Editor

eXon PUBLICATIONS

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FOREWORD

There are ~18,000 new cases of glioma diagnosed in the USA alone and their incidence has been growing; they represent up to 33% of all primary brain tumors. Around 13,000 of patients with malignant gliomas die every year and the ratio of incidence vs. mortality is indicative of the substantial challenge that gliomas present in medical practice. Aside from their impact on survival, gliomas, by virtue of their site of origin and growth characteristics, also have the potential to profoundly influence elemental capabilities such as movement, thought, speech and attention. As such, these tumors produce disproportionate effects on health and well-being of afflicted individuals.

Gliomas arise from all three types of cells supporting neurons in the brain or spinal cord: astrocytes, oligodendrocytes and ependymal cells. These different lineages produce characteristic appearances when examined under classic light microscopy, the traditional method of diagnosing different types of glial neoplasms. Most recent classification schemes, however, have been based on both histological and molecular criteria—the latter also allowing for new insights into pathogenesis and also novel, targeted therapies.

The last several decades have produced and accelerated our collective understanding of glioma etiology along with the genetic and molecular underpinnings of these diseases. Unfortunately, these insights have yet been translated into noteworthy clinical benefits for patients. One of the major and relatively recent discoveries of some significance came with the identification of mutations in the isocitrate dehydrogenase (IDH) 1 and 2 genes. This and other findings now point to the importance of metabolism in determining both the aggressiveness and therapeutic susceptibility of various gliomas. However, despite improved prognostic methods based on advanced molecular and biochemical analyses, longstanding therapies—surgery, radiation therapy, and chemotherapy—remain the mainstay of gliomas treatment, with occasional other adjunctive modalities like TTFields or Avastin. It is thus quite apparent that a magnified effort is needed in order to unlock other genetic/metabolic pathways in gliomas and exploit novel scientific insights to invent/apply new approaches to their treatment.

This book touches upon several critical aspects of glioma research and clinical therapies. The contributors represent a wide range of expertise from different disciplines. There is a considerable emphasis here on translational efforts ranging from pre-clinical investigations to clinical studies. Several chapters discuss metabolic processes in gliomas as potential therapeutic targets with specific examples of drug candidates currently under evaluation. Another aspect discussed by other authors is the issue of genetic and molecular information leading potentially to better prognostication and integration of pre-clinical knowledge with practice

to promote enhanced patient outcomes. As such, this book will likely be interest to a wide audience seeking more information on challenges gliomas present to both scientists and clinicians.

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PREFACE

Gliomas are the primary brain tumors of the central nervous system. They arise from the glial and other cells and are categorized into 1) astrocytomas, including astrocytoma, anaplastic astrocytoma and glioblastoma, 2) ependymomas, including anaplastic ependymoma, myxopapillary ependymoma and subependymoma, and 3) oligodendrogliomas, including anaplastic oligodendroglioma and oligoastrocytoma. The prognosis, especially for high-grade gliomas, is dismal; the treatment of these tumors represents an unmet need in medicine. More patients die from malignant gliomas than, for example, from melanoma. Unlike for low-grade glioma, modest progress has been made in the treatment of these tumors during the last several decades.

In this book several critical issues pertinent to the understanding and treating gliomas are discussed. The need for more clinically relevant models for studying both the disease's etiopathogenesis and the effect of treatments is urgent. The presence of glioma stem-like cells and their role in tumor progression and resistance to therapies need further documentation. Metabolism is now considered as one of the most promising targets in cancer therapies and gliomas are not different. In addition, specific mutations in metabolic pathways have become hallmark of gliomas, such as IDH mutations. There are several drug candidates under development aiming at abnormal metabolic processes and which ones have the best future remains to be seen. Personalized medicine requires not only specific targets, but also determination of who will be the best responder to therapy. Here comes the role of bioinformatics in the analyses of large amount of generated data. Neurosurgeons now receive much help through neuro-functional monitoring in order to perform precise and least damaging operations. One of the frequent symptoms of gliomas, such as epileptic seizures, is being better understood with a hope for more targeted and effective interventions.

The 13 chapters of the book tackle the above-mentioned areas of investigations and research interest. It is not possible to cover comprehensively such a big subject as gliomas in one book, but the individual contributions provide a glimpse on the magnitude of challenges and potential solutions in a variety of research areas. It is hoped that the book will be an informative step in further studies of the much-to-understand diseases like gliomas.

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Pre-Clinical Models and Potential Novel Therapies for Glioblastomas

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Abstract: Glioblastoma is an extremely aggressive form of cancer most commonly derived from neural stem cells, astrocytes, and oligodendrocytes that occurs in the brain and has a 5-year survival rate of 6.7%. These gliomas have an incidence of 3.19 cases per 100,000 person and are assigned grade IV according to the World Health Organization classification of brain tumors. Current approved therapies include surgical resection followed by a combination of radiation and chemotherapy with temozolomide, and more recently tumor-treating (TT) fields. However, there are many limitations with the current treatment strategies due to several resistance mechanisms varying from low concentrations of chemotherapeutics crossing the blood brain barrier to increased risk of temozolomide resistance in a sub-set of patients. In recent years, novel therapies and delivery systems have been developed to overcome these limitations. In this chapter, we discuss pre-clinical assessments and the evaluation of potential, promising therapeutics in xenograft models for glioblastoma using advanced magnetic resonance imaging techniques.

Keywords: blood-brain barrier; ELTD1; GL261; OKN-007; pre-clinical mouse models for glioblastoma

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INTRODUCTION

Glioblastoma is the most common form of malignant primary brain tumor and accounts for approximately 45.2% of all malignant primary central nervous system (CNS) tumors (1). These tumors are derived from three cells of origin such as neural stem cells, astrocytes, and oligodendrocytes, and have an annual incidence of 6 per 100,000 diagnosed cases in the United States (2, 3). The incidence of glioblastoma increases with age, with patients aged 75–84 having an incidence of 15.03 per 100,000 (4). The average survival for patients is approximately 12–15 months, and relative survival is extremely low, with less than 5% of all patients surviving 5 years post-diagnosis, with this value decreasing to 2% among patients aged 65 years or older (5). Glioblastomas are highly infiltrative and invasive, however, metastasis outside of the CNS is rare. The current treatment method includes surgical resection to remove the bulk of the tumor followed by a combination of chemotherapy with temozolomide (TMZ) or bevacizumab and radiation. Glioblastomas are characterized by their heterogeneity, which poses an important challenge for the development of new drug therapies (5).

To date, no correlation has been found between the development of glioblastoma and smoking or exposure to other carcinogenic agents (4). While the risk factors for glioblastoma development are not well defined, males are predisposed (1.6:1) (1). The most common class of glioblastomas are primary, representing ~90% of all cases, and most occur in older adults (mean age of 65) without any evidence of precursor lesions (6). Primary glioblastomas are characterized by overexpression of the epidermal growth factor receptor (EGFR), loss of heterozygosity on chromosome 10q, phosphatase and tensin homolog (PTEN) mutations, and lack of isocitrate dehydrogenase 1 (IDH1) mutations (7). EGFR signaling is crucial for the survival, proliferation, migration, and differentiation of all types of cells in the CNS (8). In glioblastomas, EGFR deregulation can be achieved through various mechanisms such as overexpression of the receptor/ligand and receptor mutation (8). Loss of chromosome 10 is the most frequent genetic alteration (80–90%) that occurs in glioblastoma. Loss is either entire, or of either the long or the short arm. The PTEN gene is located at 10q23.3 and is crucial for regulating metastasis and invasion (1). This tumor suppressor gene is mutated in 20–40% of glioblastomas and is most commonly seen in advanced stages of primary glioblastomas (1, 9).

On the other hand, secondary glioblastomas (~10% of remaining cases) occur in younger patients with a mean age of 45 years, and most commonly develop from lower-grade gliomas (usually astrocytoma or oligodendrogliomas) (1). This type of glioblastoma is characterized by their mutations in TP53, loss of chromosome 1p/19q, as well as IDH1/2 mutations (7). Secondary glioblastomas have also been reported to have longer overall survival when compared to primary (1). TP53 mutations occur in approximately 60–70% of all secondary glioblastomas, and is a regulator of cell cycle and apoptosis (10). Isocitrate dehydrogenase (IDH) is an enzyme in the Krebs cycle (11). The two forms of IDH (IDH1 and IDH2) are NADP-dependent and are most commonly mutated in secondary glioblastomas (10). Various studies have shown how TCA cycle gene mutations contribute to both cancer development and progression (10). IDH1/2 mutations cause an overexpression in both the vascular endothelial growth factor (VEGF)

and hypoxia-inducible factor 1α (HIF- 1α) which promotes tumor progression and invasion (10).

THERAPY-LIMITING FACTORS FOR GLIOBLASTOMAS

There has been little progress made on stand-alone therapeutics in glioblastomas. Numerous clinical trials aimed at treating both primary and recurrent glioblastomas have failed due to various reasons, including ineffectiveness at improving survival and toxicity issues. Heterogeneity continues to be a barrier for therapeutics as each subpopulation of glioblastoma cells have varying degrees of aggressiveness, growth, and therapy resistance (12). In addition to the heterogeneity of the glioblastoma cells, the tumor can be divided into three different regions (Figure 1). The tumor core is described as an area of high proliferation, inflammation, and increased incidence of necrosis (13). Along the boundaries of the tumor core exists the tumor interphase. This area is classified as the transition area between the necrotic core and the outside periphery (13). The interphase has a mild hypoxic environment while maintaining a high proliferation rate (13). Clinicians most commonly try to resect out as much of the tumor core and the interphase as possible, to try to combat glioblastoma (13). However, complete resection of the periphery is difficult as the glioblastoma cells are too diffuse to completely ablate (13). The periphery cells however, have a low proliferation index and have a higher $MGMT^-$ cell population that causes these peripheral tumor cells to be more sensitive to TMZ treatment (13).

In addition to the three different regions of the tumor, there are various other challenges to developing effective treatments for glioblastomas. For example,

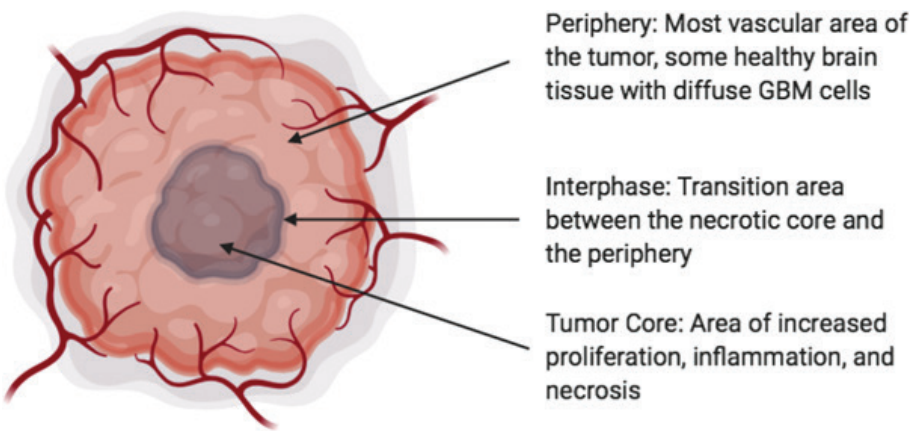

Figure 1. Description of the tumor. The tumor core is the innermost section of the tumor that is characterized by having a high incidence of necrosis along with increased proliferation and inflammation. The transition area between the tumor core and the periphery is the area known as the interphase. The periphery region includes the healthy brain tissue that has some diffuse glioblastoma cells. (Modified figure from (13); cartoon made with BioRender)

most gliomas occur in the frontal and temporal lobes (25.8% and 19.7%, respectively) while occurrences in the brainstem and the spinal cord are relatively rare (14). The most common symptoms of glioblastomas are loss of vision, numbness, and headaches, usually accompanied by nausea or vomiting (15). These symptoms may be overlooked or mistaken for another disease, which is why glioblastomas are commonly detected in later stages when they begin their widespread infiltration of the brain (15, 16). Once the tumors have developed, 60% of patients experience short seizures, between 2–3 minutes of duration, and suffer from fatigue, confusion, and numbness once the seizure has concluded (16). Neurological deficits such as vision abnormalities, speech problems, and/or loss of motor control, may also be present depending on the location of the tumor (16). Conversely, general symptoms such as personality and mood change may present as primary symptoms, causing patients to take longer to seek medical attention (16).

Another key hurdle in drug development is the blood brain barrier (BBB). This is a highly regulated semipermeable barrier that controls the movement of ions, molecules, and cells between the blood and the CNS (Figure 2). The BBB is crucial in regulating CNS hemostasis in order to protect the CNS from external toxins and pathogens and dysfunction of the BBB leads to ion dysregulation and entry of immune cells that may cause neuronal degradation (17). Endothelial cells form

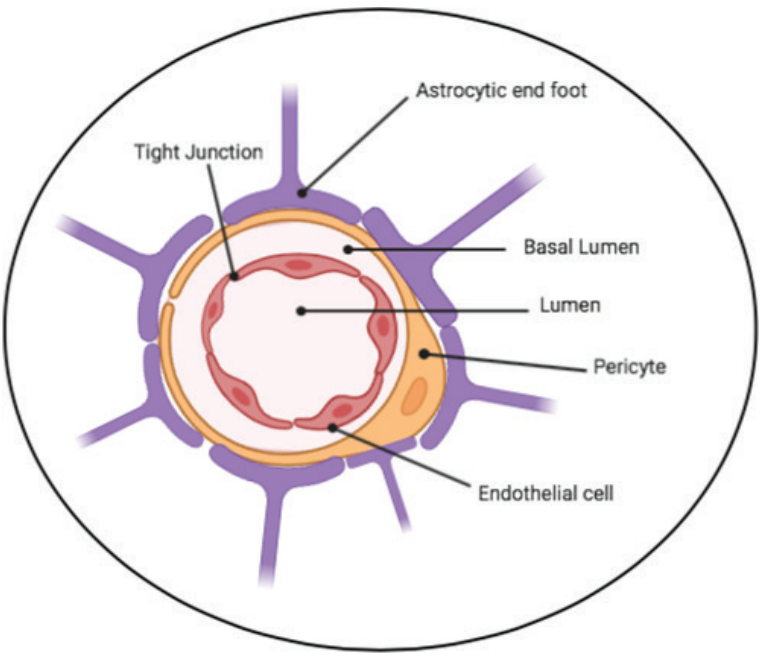

Figure 2. Graphic representation of the BBB. The endothelial cells line the lumen to form the BBB. The pericytes (shown in orange) and astrocytic feet (shown in purple) are key in keeping the tight structure of the BBB. The cartoon is modified from a BioRender BBB template.

the BBB and are connected via tight junctions (17). Pericytes are important for regulating vascular function and vessel remodeling (18). In addition, pericytes and astrocytic feet help maintain the structure and rigidity of the BBB; pericyte-deficient mutant mice were shown to have increased BBB permeability as opposed to wild-type (18, 19). Astrocytic feet wrap around and cover the majority of the outermost surface area of the BBB, and in addition to aiding in the structure of the BBB, they are also crucial in regulating the signaling pathways that help retain the junction complexes such as the tight junctions (18). There are various factors, such as lipophilicity, molecular weight and charge that influence a molecule's capacity to permeate the BBB (17, 18).

Brain tumors, such as glioblastomas, can disrupt the integrity of the BBB which increases vessel permeability allowing immune cells, plasma, and fluid to leak into the tumor regions. In glioblastoma, BBB disruption is most commonly determined via contrast-enhanced magnetic resonance imaging (MRI) by administering gadolinium-based contrast agents. This hydrophilic contrast agent accumulates within the extracellular space, and this area is then enhanced on T1-weighted (T1W) images (20). Additionally, regions of non-enhancing edema that show dysfunction or permeability of the BBB can be detected by T2-weighted (T2W) or T2 fluid attenuated inversion recovery (FLAIR) MRI (20). However, while T1/2 weighted images may show the general qualitative analysis of the BBB, they fail to show the true extent to which the BBB is disrupted in each patient, as it can vary both from patient to patient and from region to region in the same tumor (20).

PRECLINICAL MODELS

Although there is a clinical treatment plan in place, the prognosis for glioblastoma is still dismal. Preclinical mouse models are necessary to study the biology of this tumor and further identify and evaluate new potential therapies (21). Mice are most commonly used for glioblastoma research due to their accessibility and lower cost. Additionally, it is relatively easy to genetically manipulate mice, and they have short breeding times (22). Currently, glioblastoma preclinical mouse models are classified into three main categories: xenografts, genetically engineered (GEMMs), and syngeneic mouse models (Figure 3).

In xenograft mouse models, human tumor cells are transplanted either subcutaneously, or orthotopically into the brain with stereotactic surgery. Although subcutaneous injections have been widely used, these tumors lack the brain microenvironment, and therefore cannot be used to fully study the behavior of the disease (22). On the other hand, cells that are transplanted into the organ of origin allow for proper brain infiltrative behavior. Another advantage of orthotopic xenografts is that tumor sizes and sites are more consistent (22). The human tumor cells are transplanted into immunocompromised mice to ensure that the mouse does not reject the human cells (23). Some common and readily accepted immunocompromised mice are athymic nude mice, severely compromised immunodeficient mice (SCID), or non-obese diabetic (NOD)/SCID mice. Athymic nude mice are genetically modified to either have a deteriorated or absent thymus. This causes the animal to be unable to produce mature T cells, and have a reduced number of circulating lymphocytes (24). SCID mice are commonly used to

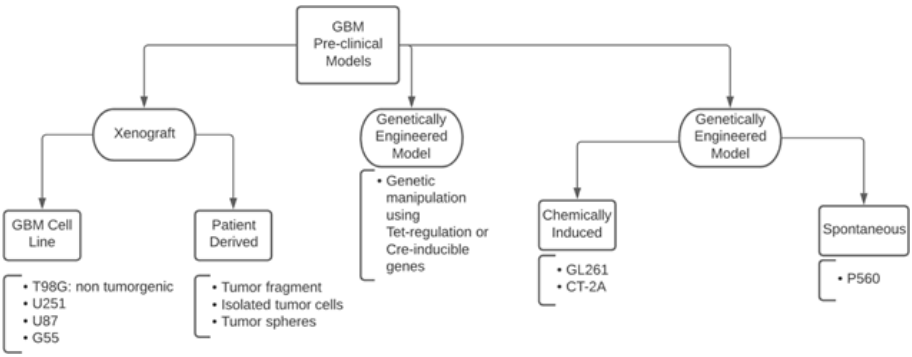

Figure 3. Summary of GBM Pre-clinical Models. A breakdown of the different classifications of the common GBM pre-clinical models found in this chapter.

research various cancers along with other human diseases because SCID mice lack B and T cells in the thymus, spleen, and lymph nodes (25). In addition to the SCID background, NOD/SCID mice have deficient natural killer cell function (26).

The xenografts can be further subclassified into either glioblastoma cell-line or patient-derived xenografts. The results of drug therapy studies from both glioblastoma cell-line or patient-derived xenografts can be obtained in weeks. Glioblastoma cell-lines are commercially available, and usually have high engraftment and growth rates (21). Additionally, glioblastoma cell-lines have higher predictability and more reliable growth/progression of the tumors. Some of the most commonly used glioblastoma cell-lines for *in vitro* and *in vivo* research include T98G, U251, and U87. The T98G cell line was generated from a 61-year old male and is characterized as having a high expression of actin alpha 2 (ACTA2) which is key for cell motility (27). However, T98G cells are not tumorigenic in mice (27).

Both the U251 and U87 established cell lines are commercially available (American Type Culture Collection-ATTC). The U251 cell line was generated from a 75-year old male with astrocytoma, and the U87 cell line was obtained from a 44-year old female (28, 29). Both of these cell lines were generated in the 1960s (28, 29). Hematoxylin and eosin analysis of U251 tumor tissue has shown characteristic tumor cell infiltrative pattern and areas of necrosis similar to that seen in human glioblastoma (30). Additionally, U251 tumors have high cell proliferation levels with over 50% Ki-67 positivity staining (31). Although the U87 cell line is one of the most widely used models with over 2,000 entries in PubMed over the last 5 years, the U87MG cells have other characteristics that are not commonly associated with glioblastomas such as having non-diffuse infiltrative patterns (30). In fact, when the U87MG cell line (obtained from ATCC) is compared to the original tumor, the DNA profile is drastically different (32). This suggests that the U87MG cell line commonly used today is a completely different version as the original tumor taken in the 1960s (32).

This begs the question, why continue using glioblastoma cell-lines if they do not mimic the true biological nature of glioblastomas? Although the U87 cell line

is not characteristic of glioblastomas, there are other glioblastoma cell lines that still remain true to the disease. For example, a less commonly used cell line, with only 33 entries in PubMed.gov (National Institutes of Health National Library of Medicine) in the past 5 years, is the human G55 cell line. These cells were originally taken from a 65-year-old male with an anaplastic astrocytoma and have displayed both tumorgenicity in nude mice and classic glioblastoma behavior with respect to aggressive proliferation, angiogenesis, and migration (33, 34).

Patient derived orthotopic xenograft (PDOX) models are created from direct implantation of either biopsied tumor fragments or freshly isolated cells without the need for intermediate cell culture (22). These models retain the genetic, histological, and molecular profile similar to the primary tumors, even after multiple cell passages (21, 22). However, a disadvantage is that PDOX models are costly and require fresh tumor fragments/cells, therefore limiting the number of facilities that can use these models. In addition to PDOX, other patient derived xenografts can be created by injecting glioblastoma tumor spheres, also known as neurospheres. Neurospheres are produced by cultivating neural stem-like cells from primary brain tumors that can then be transplanted into immunodeficient mice to form gliomas (35, 36). These tumor spheres also have the ability to retain tumor heterogeneity and specific aspects of tumor growth, such as tumor angiogenesis and cell invasion, similar to the patient's original tumor (37, 38). However, a disadvantage of this model is that not all tumors are able to be successfully cultured as tumor spheres.

GEMMs, on the other hand, have their genetic profile altered to express one or several mutated, deleted, or overexpressed genes that contribute to the malignancy that better recapitulate the molecular evolution of GBMs (23). Commonly, the GEMMs that have been established use a combination of tumor suppressor inactivation (p53 and/or Rb) and the activation of receptor tyrosine kinases (RTK) and Rb signaling, most commonly though PTEN or Nf1 deletion (39). For example, an established glioma model uses a conventional knockout of Nf1 and p53 to develop tumors is approximately 92% of the animals by 6 months (40). It is key to note that some of the mutations in key genes are lethal and therefore some GEMMs must be generated using Tet-regulation of Cre-inducible gene alleles in order to express/inactivate certain genes at specific time points (22). The tumors that result from GEM models are usually composed of homogeneous genetic change and therefore, do not reflect the heterogeneity of human glioblastoma (21). However, these models are extremely useful in determining and identifying various molecular events thought to be key in tumor growth and progression by manipulating particular pathways that drive brain tumor initiation. GEMMs are also an ideal system to study the effects of particular drugs on their molecular targets in an immunocompetent host. A host with an intact immune system allows for proper analysis of the interaction of the tumor microenvironment and potential therapeutics. Furthermore, GEMMs can be easily used in humanized mice studies. A major disadvantage however, is that GEM models commonly require months to a year to develop prior to starting drug therapy studies (23). Additionally, because the tumors are not of human origin, they cannot reliably mimic glioblastoma behavior and cannot reliably predict therapeutic response (23). A comprehensive description of mouse models of experimental glioblastoma is provided in chapter 2.

Lastly, syngeneic mouse models are also commonly used in glioblastoma research, due to the fact the cells can be implanted in immunocompetent animals. This model allows for proper examination of immune interactions and is commonly used to study potential immunotherapeutics (21). Within syngeneic mouse models, there are two different sub-categories: carcinogen/chemically induced and spontaneous. Carcinogen induced models include GL261 and CT-2A. GL261 was the first and is the most commonly used immunocompetent mouse model. These cells can be implanted and form tumors either in the subcutaneous region or intracranially (41). However, there is some concern that the GL261model has also drifted genetically and does not authentically model glioblastoma characterizations or attributes. Examination of GL261 characteristics showed a mutation in KRAS, an oncogene mutated in 25% of all tumors, and may affect how different therapeutics respond (41). Additionally, for the CT-2A models, 20-methylcholanthrene pellets were intracranially implanted into C57/BL6 mice and the subsequent astrocytoma formed was used to establish the cell line (42). Similar to classical GBM characteristics, CT-2A tumors are deficient in PTEN protein, are necrotic and chemo-resistant, and undergo unregulated angiogenesis (38). While spontaneous models, such as P560, best reflect the natural spontaneous nature of human GBM disease, large cohorts of animal are necessary, and the cost may outweigh the positives (38).

With advancements in science, we can now monitor the transplanted tumor cells and tumor growth *in vivo*. One way to achieve this is by expressing a luciferase cassette in the tumor cells so that movement of the cells and growth of the tumor can be monitored by bioluminescence. However, a disadvantage to this method is that it commonly requires a larger number of cells to be injected, which may have an impact on the tissue architecture (22). Another method is MRI, which allows monitoring of tumor growth and progression, along with various other aspects of tumorigenesis such as perfusion imaging.

Pre-clinical mouse models have been widely used for many years. Ideal models should mimic classical histopathology, behavior, and genetic mutations as human glioblastomas as well as be reproducible. However, to date, no one model is able to completely mimic all characteristics of glioblastoma. This is because ongoing research uncovers and updates genetic and cellular mechanisms in human glioblastomas. Additionally, continuous passaging of cell lines/PDX models introduces new mutations and genetic drift over time. Therefore, further research needs to be done to establish mouse models that can fully represent human glioblastoma.

PROMISING THERAPEUTICS

To date, the current treatment plan is not effective in combating human glioblastomas. Although animals have been successfully treated with bevacizumab, as well as check-point inhibitors, the results did not translate to significant increase of survival in patients. Therefore, new therapeutic approaches are crucial. While there are various therapies being examined for glioblastomas, this chapter touches on only three promising therapies from the literature.

EGFR/HER1 in humans is a transmembrane receptor tyrosine kinase that is overexpressed in various cancers, including glioblastoma (43). Overexpression of

EGFR results in increased unregulated growth and survival of glioblastoma cells (44). While the EGFR has been an attractive drug target against glioblastomas for years, there has not been an effective therapy reported. Both first- and second-generation EGFR inhibitors (gefitinib and afatinib, respectively) successfully decreased cell proliferation, growth and angiogenesis in tumor models, however, did not have a significant effect in clinical trials (43). More specifically, gefitinib did not show an effect on survival in phase II trials of relapsed glioblastoma, and afatinib did not have an effect on survival in primary nor recurrent glioblastomas (45, 46). The failure of the first- and second- generation of inhibitors exposed the two main issues with the drug. First, in order for the drug to have an effect, sufficiently high concentrations of the drug are required (43). Secondly, the EGFR inhibitors are unable to successfully cross the BBB (43). As stated above, the BBB of patients is not fully disrupted, and the extent of disruption differs from patient to patient and from region to region within the same tumor (43). Therefore, in order for an EGFR inhibitor to be successful, the new drug must penetrate the BBB. Osimertinib (AZD9291) is currently used to treat lung cancer and is an oral, third generation irreversible EGFR inhibitor (43, 47). Studies have shown that AZD9291 can penetrate the BBB, inhibit tumor growth, and may be effective as a brain tumor therapeutic (47). When comparing AZD9291 to gefitinib, the concentration of AZD9291 in the brain can reach up to 10-fold higher than gefitinib (43). Tumor heterogeneity is also an important factor to consider when targeting tumors.

Unregulated angiogenesis is critical for the maintenance of the tumor, as the newly formed blood vessels deliver nutrients deep inside the tumor core. To date, the main angiogenic pathways have been Notch/DLL4 and the vascular endothelial growth factor (VEGF). The notch signaling pathways have been highly conserved throughout various species and are crucial for multiple aspects of tumorigenesis (cell proliferation, migration, and tumor angiogenesis) (48, 49). Similarly, VEGF promotes tumorigenesis and angiogenesis (34). The epidermal growth factor, latrophilin and seven transmembrane domain-containing protein 1 (ELTD1, alternatively known as ADGRL4) is an angiogenic biomarker. In normal vasculature, the expression of ELTD1 is increased by VEGF, and decreased by the Notch/DLL4 signaling pathway (50). ELTD1 is overexpressed in human high-grade gliomas, when compared to low grade gliomas, and when inhibited through varying antibodies (polyclonal, monovalent monoclonal, and single chain variable fragment (scFv)) effectively decrease tumor volumes, completely normalize the tumor associated vasculature and increase survival (34, 51, 52). RNA-sequencing analysis also revealed that anti-ELTD1 therapy may have an effect on other aspects of tumorigenesis such as migration, cell proliferation, and invasion (34). While the BBB has been an issue for some potential therapies, molecular targeted MRI showed that an optimized scFv antibody treatment against ELTD1 was successful in reaching extremely diffuse tumor regions that were otherwise undetectable via conventional MRI (51). In a G55 xenograft mouse model, anti-ELTD1 treatment was also found to reduce and target two angiogenic pathways, VEGFR2 and Notch (34, 51, 52). Additionally, unlike Bevacizumab, an anti-angiogenic therapy, anti-ELTD1 treatment had no signs of hemorrhaging in a pre-clinical mouse model (34). Further analysis is needed to determine if anti-ELTD1 therapy would be effective in clinical trials. Ongoing research in our group is assessing the ability of anti-ELTD1 antibody therapy against other aspects of tumorigenesis (e.g. cell

proliferation, cell invasion and apoptosis), as suggested from RNA-seq data in our previous pre-clinical study (34).

OKN-007 (OKN) is a small molecule that can cross the BBB and specifically affects the transforming growth factor β1 (TGF β1) (53). OKN has been widely studied in various glioma models (C6, U87, F98, GL261, and G55) and is currently in two glioblastoma clinical trials. The first is a phase II clinical trial of OKN-007 combined with TMZ in patients that have recurrent glioblastoma, while the second is an early phase I trial that looks at the side effects of OKN-007 with TMZ in patients that are undergoing concomitant radiotherapy after surgery (54). OKN-007 significantly affects every aspect of tumorigenesis by decreasing cellular proliferation, migration, angiogenesis, and increasing apoptosis (55–57). Treatment with OKN resulted in increased survival, decreased tumor volumes (as measured by MRI), and inhibition of tumor necrosis (as measured by MRI – morphological and diffusion-weighted imaging, MR spectroscopy, and histology) (57–59). Regarding angiogenesis, OKN decreased both VEGFR2 and HIF-1α protein expression (56, 59). Additionally, OKN and TMZ combination treatment significantly increased percent survival, decreased tumor volumes, and normalized the vasculature in TMZ resistant glioblastoma cell lines (55). RNA-sequencing studies have also shown that OKN-007 also has an effect on 57 genes associated with the extracellular matrix through TGFβ1 including collagens and MMPs (55). Altogether, this suggests that OKN-007 may be effective in targeting multiple aspects of tumorigenesis. To date, no dose-limiting toxicities, nor adverse side effects, were observed with OKN-007 in an initial phase Ib/IIa clinical trial (60). Currently, OKN-007 is being investigated in an ongoing multi-institutional phase II clinical trial.

CONCLUSION

There have been numerous drug therapies proposed for the treatment of glioblastoma. However, many of those proposed therapies have failed due to various reasons, ranging from inability to penetrate the BBB to failure to translate significant results in human trials. Here we discussed three different promising therapies that are able to bypass the BBB and have the ability to hit multiple tumorigenic pathways. Glioblastomas are complex heterogeneous tumors that have the ability to adapt and build resistance against existing treatments. This therefore prompts the need for new therapies that have the capacity to target various pathways so that they remain effective against all subpopulations in the tumor.

Conflict of Interest: Dr. Towner holds patents regarding the use of OKN-007 and anti-ELTD1 antibody therapy in gliomas. Ms. Zalles declares no potential conflicts of interest with respect to research, authorship, and/or publication of this chapter.

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2

Mouse Models of Experimental Glioblastoma

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Abstract: Glioblastoma is one of the most common malignant brain tumors. It has poor prognosis: the survival rate is 14–15 months, even with treatment by surgery, radiation, and chemotherapy. To develop more efficacious therapies, it is essential to generate preclinical mouse models that enable mechanistic studies. Multiple murine glioblastoma models have been generated, each with distinct advantages and disadvantages. The traditional Cre-LoxP system specifically targets glioblastoma-related genes but requires extended experimental timelines. CRISPR-Cas9 methods require less time to generate mouse models, yet the off-target effects lead to variable glioblastoma phenotypes. Transposon-based insertional mutagenesis models can intercept and promote transcription but has strict limitation of insertional transgene size. Allograft cell line injection into immunocompetent mice prevents immune rejection but fails to recapitulate various features of human glioblastoma. Intracranial injection of patient-derived xenograft cell lines into immunocompromised mice preserves features of human glioblastoma but does not allow the study of immune cell function in preclinical immunotherapeutic approaches. Finally, humanized mouse models offer the potential to analyze the human adaptive immune response but not the innate

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immune response. This chapter outlines the major experimental glioblastoma models currently employed and the therapeutic approaches that can be tested.

Keywords: Cre-LoxP glioblastoma model; CRISPR/Cas9 glioblastoma model; mouse models of glioblastoma; transgenic glioblastoma model; transplant glioblastoma model

INTRODUCTION

In 2016, WHO integrated histological and molecular parameters to define the main gliomas types in place of the previous criteria taking into account only histology (1). Under the new criteria, based on histological features, gliomas are classified into four grades. Grade I is the slow growing, less malignant tumors; grade IV is the rapidly growing, highly malignant tumors (2). Glioblastoma is the most aggressive and invasive undifferentiated tumor type and has been designated Grade IV by WHO (3, 4).

Based on the molecular feature of whether there is isocitrate dehydrogenase (*IDH*) mutation, glioblastomas are mainly classified as *IDH*-wildtype and *IDH*-mutant (3, 5–7). *IDH* mutant glioblastomas are molecularly, biologically, and clinically different from *IDH* wild-type ones (5), which is important for glioblastoma biology and heterogeneity (8, 9). Clinically, primary glioblastoma cases are more related to *IDH*-wild type; secondary GBM cases are more related to *IDH*-mutant type; approximately 75% of patients with secondary glioblastoma have *IDH* mutation (10).

Pertaining to molecular gene expression studies, there are three major genetic pathways related to glioblastoma formation: (i) inactivation of the *p53* pathways accounts for 87% of glioblastomas; (ii) inactive retinoblastoma (*RB*) tumor suppressor pathways account for 77% of glioblastomas; and (iii) amplification and mutation of receptor tyrosine kinase (*RTK*) genes and activation of the phosphatidylinositol-3-OH kinase (*PI3K*) pathways account for 88% of glioblastomas (11, 12).

Finally, *in vivo* experiments have demonstrated that most GBM tumors exhibit deregulation and mutations of genes in the *p53*, *RB* and *RTK/RAS/PI3K* pathways (13–16). Clinical therapies targeting these pathways are being developed, but the treatments have not been successful (17–20) due to inefficient blood brain barrier penetration, inter-tumor heterogeneity and other compensatory/redundant signaling pathways. To better understand those pathways and their interplay, there is a need for models that reflect the glioblastoma tumor microenvironment (TME), because current *in vitro* models are not able to recapitulate this. Traditionally, 2D monolayer cell lines cultured in serum-containing medium do not reflect the heterogeneity of human tumors, and hence do not resemble clinical tumor development. Newer approaches using 3D spheres brain cell culture, such as glioma stem-like cell culture, in serum-free medium can reflect better genetic background of the tumor and maintain some phenotypic heterogeneity. However, long-term culture results in the clonal selection and genetic drift. Furthermore, *in vitro* cell culture does not model human immune cells. This limits exploration of factors regulating tumor-host interactions and immune control (21–23).

Therefore, it is essential to have animal models that properly reflect the glioblastoma TME so that the glioblastoma biology can be precisely analyzed, which allows for the evaluation of potential treatments, immune therapies and identifying the therapy targets.

MOUSE MODELS OF GLIOBLASTOMA

Currently, there are four major strategies for generating glioblastoma mouse models: spontaneous, transgenic, transplant, and humanized (Table 1).

Spontaneous glioblastoma mouse models

Spontaneous mouse glioblastoma tumors are rare (24). Therefore, setting up the spontaneous glioblastoma mouse model would require a large number of mice to observe. Slye, Holmes and Wells found only 3 spontaneous glioblastomata out of 11,188 mouse brains (25). To increase the efficiency and speed of spontaneous tumor generation, chemical or viral induction methods were used. The first successful induced brain tumor was developed in 1939 with intracranial implantation of 20-methylcholanthrene into C3H mice subarachnoid by Seligman and Shear, which led to gliomas and meningeal fibrosarcomas (26). Even though chemical-induced glioblastoma models are now outdated, several mouse cell lines established from those tumors have been later used for allograft implantation mouse models (27–31). Rous sarcoma virus has been used to induce mouse glioblastoma since the 1960s (32–34). However, virus-induced tumors had incomplete tumor penetrance (35, 36). The special maintenance requirement of the virus and the virus-induced mice dramatically increases the cost. In recent years, engineered viruses as vectors for transgenic genes are now used to generate mouse glioblastoma models. Both retrovirus and lentivirus have been applied this way, as will be discussed in the following section.

Transgenic glioblastoma mouse models

Currently, the main systems used for transgenic mouse models are the Cre-LoxP system, transposon-based system, CRISPR/cas9 system, and virus vectors delivery system. These systems can be used in both germline and somatic transgenic mice (37). The common goal of generating mouse glioblastoma models is increasing activity through overexpression of oncogenes such as *p21-RAS, PI3K, EGFR, CDK4* and *MDM2*, or decreasing activity by mutating tumor suppressor genes, such as *Pten, p53, CDKN2A* and *RB* (34). Generally, germline transgenic mouse models are generated by first introducing defined DNA alterations in germline cells, then using breeding strategies to obtain the gene related to the experiment by serial breeding from the founder mouse (38, 39). In somatic transgenic mouse models, tumors are initiated by directly implanting the induced transgenic cells, RNA, sh-RNA or engineered virus vectors into specific brain regions (15, 40–43). In recent years, these techniques have been combined to generate more precisely targeted mouse models for research. Fluorescence protein, luciferase reporter, or other tags such as human influenza hemagglutinin are tagged to the transgenic

TABLE 1 **Major experimental glioblastoma mouse models**

Model Types	Turnaround Time	Advantages	Disadvantages	Citation
Spontaneous mouse models	Variable	Reflects tumor development and progression	Need a large numbers of mice	
Spontaneous				(24, 25)
Chemical induced				(26)
Virus induced				(32, 33)
Transgenic mouse models		Can determine the molecular genetics alteration	Cannot completely reflect the phenotypic heterogeneity.	
Cre-LoxP system	10–14 months			
Traditional Cre-LoxP system		Stable, accurate	Only spatially control, led longer time needed for development.	(15, 46)
Tamoxifen inducible system		Temporal control		(51, 52)
Tet/Dox inducible system		Temporal control		(56, 57)
Transposon system	6–8 months	Less generation time		
SB system				(41, 73)
PB system				(75)
CRISPR/Cas9 system	5–7 months	Less expensive, faster, easy to introduce.	Off- target	(92, 94)
Viral vector delivery system	Days	Faster development	Vector has insertion limitation <2.5Kb	(90, 103)

Table continued on following page

TABLE 1 Major experimental glioblastoma mouse models (Continued)

Model Types	Turnaround Time	Advantages	Disadvantages	Citation
Transplant mouse models				
Allograft transplant models	21–30 days	Can model the immunity and immunotherapy of mouse experimental glioblastoma.	Murine immune response	(109, 115)
Xenograft transplant models	21–30 days	Reflects the genetic and phenotypical feature of original human tumor	Mouse is devoid of murine or human immune system	(117, 118)
Humanized mouse models			Cannot completely reflect human response	
Hematopoietic stem cells (HSCs) humanized mice	10–12 weeks	Provide fully competent human immune system		(156)
Human microbiota-associated (HMA) humanized mice	3 weeks	Avoid the impact on immune system by gut microbiota composition		(164, 165)

genes so that tracing glioblastoma growth in the mouse models, labeling targeted cells and tissue, and observing microenvironment diffusion and the immune cell response for therapy can be more convenient under the microscope (44).

Cre-LoxP transgenic glioblastoma mouse models

Most glioblastoma mouse models have used the Cre-LoxP system to target tumor genes in the specific brain tissue of interest (Figure 1A, B) (45). This system provides deep insight into the genetic drivers of glioblastoma and highlights the genetic differences between primary and secondary glioblastomas (13, 15, 46). Previously, most mouse models were generated by breeding two transgenic mice strains: a Cre-driver mouse strain which has Cre recombinase with a promoter and a LoxP floxed mouse strain that has LoxP floxed critical exons of the target gene (Figure 1C) (47). By breeding the two strains together, the system deletes the floxed region and inactivates the gene in desired tissues; the target gene remains functional in all other tissues. On average, it takes 12–18 months to obtain the desired transgenic mice. Therefore, induction of LoxP sites via Cre recombinase viruses—such as adenovirus and lentivirus—has been used to shorten the experimental timeline and generate more complex yet easy to obtain transgenic mouse models (14, 15, 37, 48).

Both of the above strategies have been applied in testing *p53* and *PTEN* function in GFAP positive glioblastoma tissues as demonstrated by the following studies. Zheng et al. generated *p53* and *PTEN* double knock-out mice targeted specifically to astrocytes by using *GFAP-Cre*$^{+}$ mice interbred with *P53*$^{flox/flox}$*;Pten*$^{flox/+}$ mice. From these, 66% of the tumors were anaplastic astrocytomas and 34% were glioblastomas (13). Their model indicated that the loss of *p53* and *PTEN* would regulate *Myc* levels and in turn control NSCs self-renewal and differentiation (13, 46). Jacques et al. demonstrated another method for generating transgenic mice that target GFAP positive cells: they used adenovirus expressing Cre recombinase (Adeno-Cre or Adeno GFAP-Cre) injected into mice that have conditional alleles flanked by LoxP sites of *RB, p53*, and *PTEN*, to ablate *RB/p53*, *RB/p53/PTEN*, or *PTEN/p53* in adult mice stem/progenitor cells. Their result indicates that initial deletion of *RB/p53* or *RB/p53/PTEN* are relevant to glioblastoma pathogenesis, and that *RB* loss is important in driving the phenotype of primitive neuroectodermal tumors (14). Friedmann-Morvinski et al. performed stereotaxic injection of Cre-inducible lentiviral vectors *shNF1-shp53* or *H-RasV12-shp53* into GFAP-Cre mice to induce *p53* deficiency in GFAP positive cells such as astrocytes. They identified that loss of *NF1* leads to increased *RAS* mitogenic signaling and increased cell proliferation, while the loss of functional *p53* induces genomic instability for glioblastoma tumorigenesis (15, 37).

Cre-LoxP has been a popular system for generating transgenic mouse for years, it can only spatially but not temporally control the tumorigenesis (49, 50). In addition, knock-out or overexpression of some critical genes may lead to early embryo lethality (51, 52). To overcome this shortcoming and accurately control the timing of tumor generation, traditional Cre-LoxP system has been modified so that it can be temporally induced by exogenous inducer tamoxifen (TAM) or tetracycline/doxycycline (Tet/Dox), making the gene expression transient and reversible (16, 53, 54).

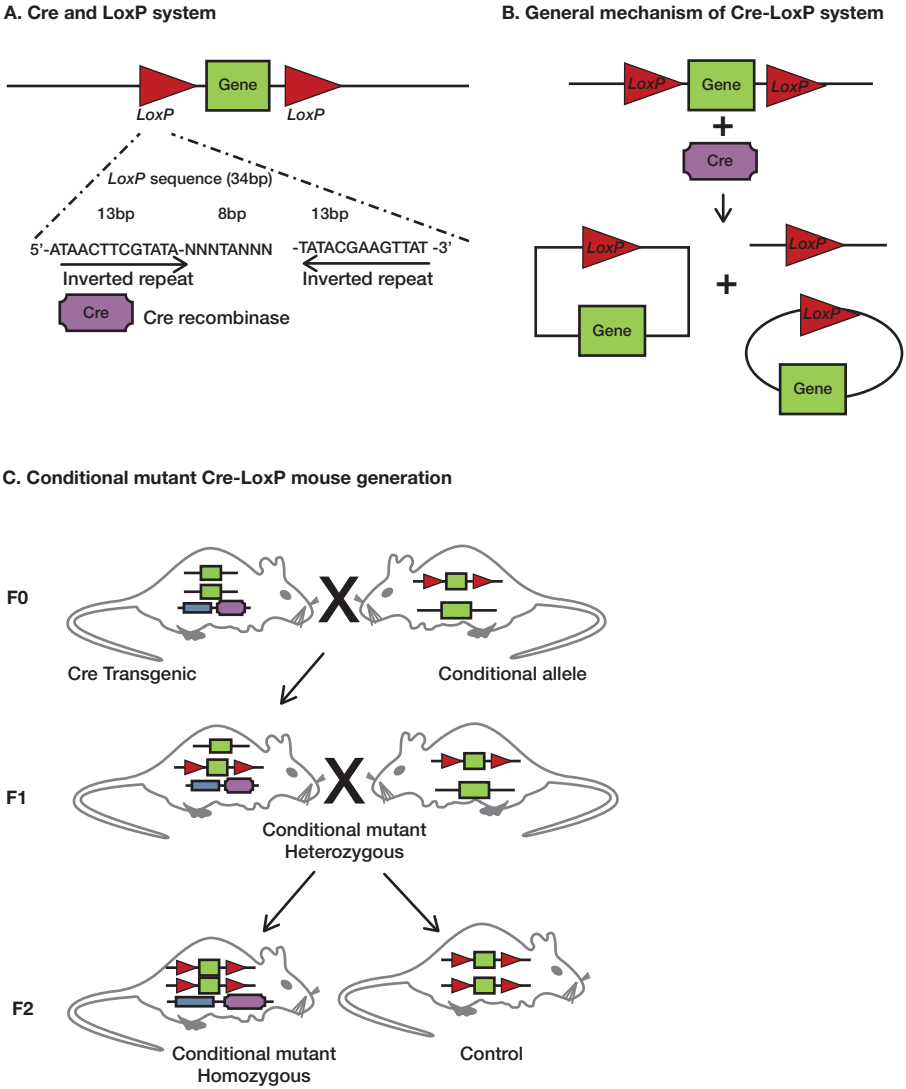

Figure 1. General Cre-LoxP deletion system. **(A)** Cre and LoxP system; 34-bp LoxP sequence consisting of two 13-bp inverted and palindromic repeats and 8 bp of core sequences; Cre recombinase is a 38-kDa DNA recombinase. **(B)** General mechanism of Cre-LoxP system; Cre recombinase recognizes the specific DNA fragment sequences between the two repeated LoxP sites and mediates site-specific deletion of DNA to create two pieces of DNA. **(C)** Traditional Cre-LoxP mouse generation;two strains of mutant mice are bred to generate Cre-LoxP mice: a Cre-driver mouse strain which has Cre recombinase with a promoter, and a LoxP floxed mouse strain that has LoxP floxed critical exons of the target gene; breeding these two strains together generates heterozygous F1 founder mice; F1 mice then breed with LoxP mice again for the F2 homozygotes.

Inducible Cre-LoxP transgenic glioblastoma mouse models

There are two widely used inducible Cre-LoxP systems. One is TAM inducible Cre-LoxP system (CreER^{TM}-LoxP system) (Figure 2 A, B) (53). Cre recombinase is fused to estrogen receptor (ER) to prevent CreER^{TM} from entering the nucleus and driving the floxed LoxP sites to delete the target transgenic DNA. When TAM, an ER agonist, is administered into the CreER^{TM}-LoxP system, it binds to the ER and initiates the translocation of CreER into the nucleus, where it can recombine with the floxed LoxP target exon of the DNA. Thus, it can control the timing of gene expression or inactivation and be used to overcome the limitation of Cre-LoxP system where some loss/gain of gene functions would lead to the lethality of mouse in embryo stage or early young (51, 52). CreER^{TM} is Cre recombinase fused to one mutated human ER. This CreER^{TM}-LoxP system needs a higher TAM dosage for induction. To avoid the potential side effects of high TAM levels, CreER^{T2} was generated. It consists of Cre recombinase fused to a triple mutant form of the human ER. Thus, only 1/10 of the TAM dosage required for the CreER^{TM} system is needed to activate CreER^{T2} (55).

To generate CreER^{TM}-LoxP inducible germline transgenic mouse models, two independent strains of mice are required. One strain expresses CreER^{TM} controlled by a cell-specific promotor. The other expresses floxed LoxP sites. The two strains of mice are bred together to generate the double transgenic mice. Adding TAM to the mice's food or drinking water permits spatiotemporal control of the target gene expression. This method efficiently avoids early embryos lethality. *IDH1* knock-in mice died perinatally if crossed *IDH1*$^{fl(R132H)/+}$ mice with *Nes*-Cre mice (51, 52). Bardella et al. successfully generated live *Nes*-CreER^{T2}; *IDH1*$^{fl(R132H)/+}$ knock-in mice by crossing *IDH1*$^{fl(R132H)/+}$ mice with the TAM-inducible *Nes*-CreER^{T2} mice. At 5–6 weeks of age, TAM induction was performed for 5 consecutive days to successfully obtain *R132H* knock-in mice. This mouse model demonstrates that overexpression of *IDH1* mutation in mouse brain subventricular zone (SVZ) cells contributed to glioblastoma formation through *Myc* and *Wnt* pathways activation, telomere pathway activation, and DNA hypermethylation (51).

The CreER^{TM}-LoxP system is extremely versatile due to the ease of gene expression control it provides. For example, brain progenitor cell specific inducible Cre mice *Ascl1*-CreER^{TM}, *NG2*-CreER^{TM}, and *Nes*-CreER^{T2} were crossed with knock-out or conditional knock-out *NF1*, *p53*, and *PTEN* mice to generate double transgenic CreER^{T2} floxed LoxP mice. Then the mice were induced by TAM at 4–8 weeks of age, and the timely control of *NF1*, *p53*, and *PTEN* knock-out in specific cells expressing *Ascl1*, *NG2*, and *Nes* allowed for identification of central nervous system cell lineages contributing to glioblastoma (8, 54).

The other widely used inducible Cre-LoxP system is Tet inducible Cre-LoxP system (Figure 2 C, D). Dox is an analog medicine to Tet. Since Dox is more efficient in controlling the Tet receptor, researchers use Dox more than Tet in this system. Thus, the system is also called the Dox inducible Cre-LoxP system. There are two types of Tet/Dox inducible systems: Tet/Dox-on and Tet/Dox-off, depending on whether the system uses reverse tetracycline-controlled transactivator (rtTA) or tetracycline-controlled transactivator (tTA). In Tet-on systems, addition of Tet induces gene expression. In Tet-off systems, the desired gene is expressed in the absence of Tet (38, 47). Both the Tet-on and Tet-off systems are

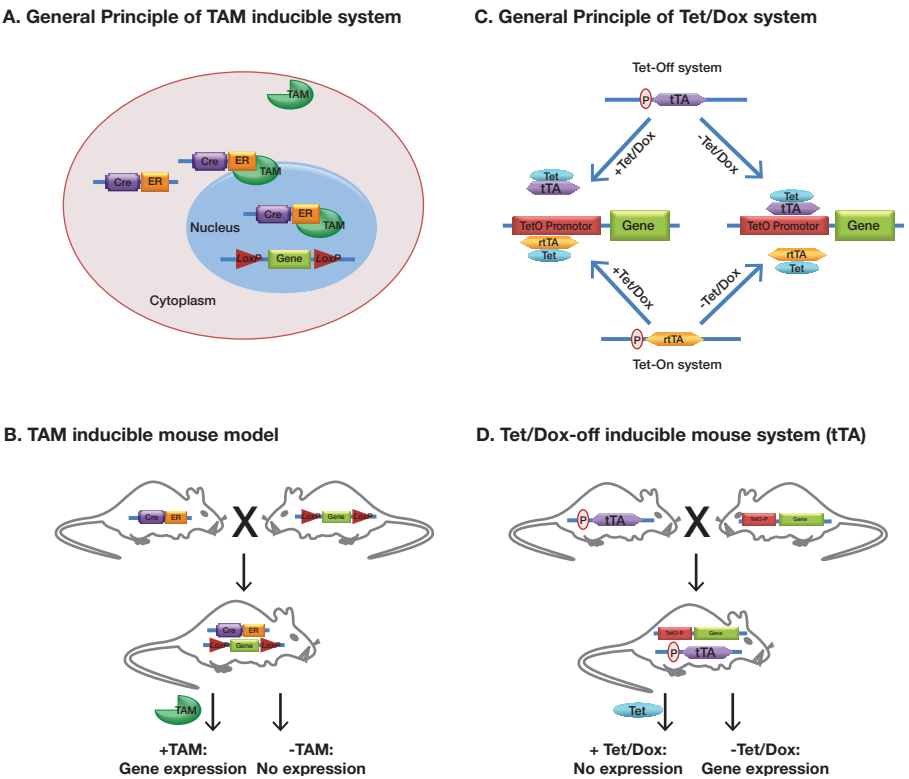

Figure 2. The Cre-LoxP inducible system. A. General Principle of TAM inducible system; in TAM inducible system, Cre is ligated to the ER which stays in the cytoplasm until the administration of TAM; when TAM is administered, CreER binds to the estrogen receptor and initiates the translocation of CreER into the nucleus, where it recombinase with the floxed LoxP target exon of the DNA. **B.** Tamoxifen inducible mouse model; two independent mouse strains, one strain expressing CreER, the other expressing two LoxP sites with or without a stop code, are bred together to generate double transgenic mice; adding TAM to food or drinking water of the double transgenic mice permits in vivo spatiotemporal control of the target gene expression. **C.** General principle of Tet/Dox system; two types of Tet/Dox general inducible systems: Tet-on and Tet-off. In Tet-on systems, rtTA is expressed; in the absence of Tet/Dox, inactivated rtTA cannot bind to TetO sequence of Cre gene, so Cre is not expressed; after Tet/Dox administration, activated rtTA binds to TetO promoter of Cre to induce Cre expression, which activates the Cre-LoxP system; In the Tet-off system, tTA is expressed; in the absence of Tet/Dox, activated tTA can bind to TetO sequence of Cre and induce Cre expression; after Tet/Dox administration, tTA is inactivated; inactivated rTA cannot bind to TetO promoter, therefore Cre expression is inhibited. **D.** Tet/Dox-off inducible mouse system (tTA); two independent strains of transgenic mice are needed: one strain requires tTA expression, the other strain requires the expression of the mutant gene of interest is controlled by TetO promotor with Cre expression; these two strains of mice are bred together to generate double-transgenic mice; by adding Tet/Dox to food or drinking water of the double transgenic mice allows the *in vivo* target gene to express spatiotemporally.

used widely to spatiotemporally control tumor generation in transgenic mouse models (16, 40).

Similar to the TAM inducible transgenic mouse model, the Tet/Dox inducible model requires two independent strains of transgenic mice. One strain is the transactivator requiring rtTA or tTA expression under the control of a specific promotor. The other is the responder requiring that the expression of the mutant gene of interest is controlled by TetO promotor with Cre expression. These two strains are bred together to generate the desired double-transgenic mice. Then Tet/Dox is added to food or drinking water of the double transgenic mice to allow the target gene to express spatiotemporally (56, 57). Although Tet/Dox inducible Cre-LoxP system can flexibly control the timing of transgenic expression, one shortcoming is the leakiness of rtTA, which can result in undesired transcription of the target genes. This is because rtTA has some affinity for TetO sequences even in the absence of Tet (58). In addition, the potential side effects from high doses of Tet/Dox are also unknown. To avoid these limitations, mutagenized rtTA^{2S} were generated to increase Dox sensitivity, allowing it to function at Dox concentration 10 times lower than rtTA (59).

Transposons-based transgenic glioblastoma mouse models

Transposons were first identified more than 50 years ago (60). Transposons can move from one genomic location to another through "cut-and-paste" mechanisms (Figure 3 A) (61, 62). Sleeping beauty (SB) and PiggyBac (PB) are two widely used transposases that have been successful in establishing functional mutagenesis *in vivo* and *in vitro*. SB transposase inserts a transposon into a TA dinucleotide base pair sequence in the recipient DNA, while PB transposase inserts a transposon into a TTAA dinucleotide base pair sequence (63). PB integration sites are mainly localized near transcriptional start sites (TSSs), CpG islands, and DNaseI hypersensitive sites. In contrast, SB integrations are more randomly distributed, so the PB system can perform more efficient stable gene transfer than the SB system (64–66). SB and PB transposon systems have been used in both germline and somatic cells of transgenic mice (Figure 3 B, C) (45, 60, 65, 67–71).

In germline transposon models, two mouse strains, one that expresses the transposase and one that carries transposons with gene trap cassettes are needed to breed the desired mice in multiple generations (60, 69, 72). For example, *Rosa26-LSL-SB11* (SBase) mice which had conditional floxed-stop SB transposase allele knocked in are bred with *T2/Onc2,3* mice which had mutant SB transposon to generate heterozygous mice that expressed SB transposase *T2/Onc2,3/+; SBase/+*. Then these mice interbred to produce homozygotes for later experiments (73).

The transposons model can also be used in the context of somatic cell mouse models. Virus SB *transposase* system was used to overcome the shortcoming of transient expression of polyethylenimine/plasmid DNA (PEI/DNA) (74). Thus, it was able to deliver *shRNA-p53* with seven other combinations to identify the functions of the oncogenes in different glioblastoma formation pathways. This technique enabled rapid production of different genetically engineered mouse strains and sped up the preclinical drug screening for glioblastomas (70, 75, 76).

The SB/PB transposase system can also avoid embryonic lethality in mice (77). For example, *ATRX* mutation, together with mutation of *p53* and point mutation of histone *H3.3* variant, occurred in 31% of primary glioblastoma in pediatric

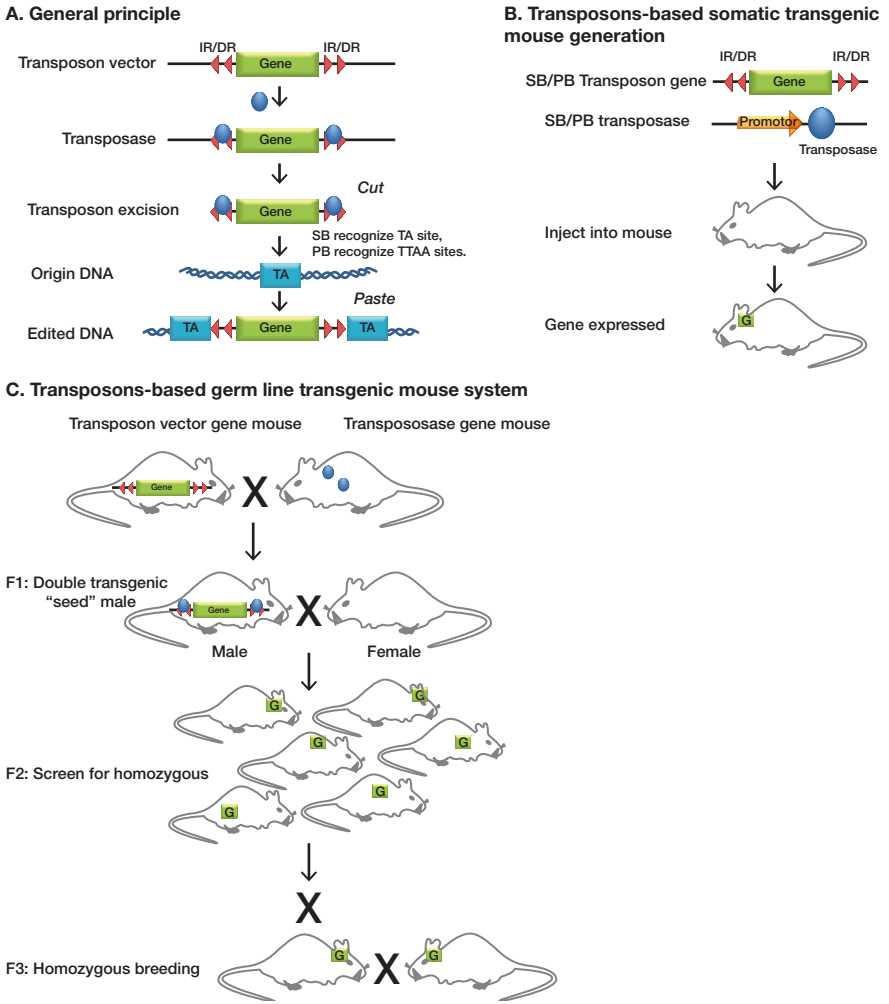

Figure 3. The Transposons-based system. A. General principle of transposon-based system; the transposon-based system includes two parts: a transposon vector containing DNA sequence that is flanked by inverted repeat/direct terminal repeat (IR/DR) sequences, and the transposase enzyme responsible for excision and reintegration of the transposon under the control of a promoter; when transposon vector and transposase are present together, a "cut-and-paste" transposition reaction occurs; the transposon is excised from its original location and re-integrated to a new location within the genome. *SB* and *PB* are two different transposases. *SB* transposase inserts a transposon into a TA dinucleotide base pair sequences, *PB* transposase inserts a transposon into a TTAA dinucleotide base pair sequences. **B.** Transposon-based somatic cell transgenic mouse system; to generate the somatic cell transgenic mouse model, two plasmids are injected together into mice to cause mutations in specifically targeted cells; the transposon insertion sites are detected using PCR screening. **C.** Transposon-based germ line cell transgenic mouse system; to generate the germ line cell transgenic mouse model, two mouse strains are required: one strain carries the transposons vector gene, and the other carries the transposase gene; these two mouse strains are bred to generate the F1 generation of double transgenic mice; F1 males are crossed with wild-type females to segregate the different insertion events in their sperm cells, generating F2 in the process; then the F2 mice are screened to select the ones with the desired mutant allele, and these mice are crossed together to generate F3 homozygous mice.

patients (78–80). However, knock-out of *ATRX* resulted in embryonic lethality in mice (75, 77); the zygotes never grew beyond the 4-cell stage (75). Koschmann et al. (41) used SB transposase system to develop somatically mutant *ATRX* in mice to overcome this limitation. They injected combined plasmids encoding SB transposase/firefly luciferase, *shRNA-p53*, and *NRAS*, with or without *shRNA-ATRX*, into the lateral ventricle of neonatal mice to generate *ATRX* deficiency, *p53* loss, and *NRAS* overexpression mouse model (41). But Pathania et al. (75) using SB system by combining $H3.3^{K27M}$-SBase with *ATRX/p53* knock-down constructs injected in neonatal mice, could not induce tumor. Then this group injected combined plasmid produced by PBase system: a transposable ***sh***RNA against *ATRX* together with $H3.3^{K27M}$, and a plasmid knock-down *p53* with CRISP/Cas9, into E12.5-E13.5 embryos to generate the desired mouse model (75, 76). In short, both Koschmann and Pathania tried to use *SB* system to generate glioblastoma models via double knock-out *ATRX* and *p53*. Koschmann et al. succeeded through the SB system in neonatal stage, while Pathania et al. failed with the SB system in neonatal stage but succeeded with the PB system in embryo stage. This suggests that the somatic transgenic stage may be more critical for the lethal genes than the choice of method.

In the SB transposon system, there is only 40–50% chance that the excised transposon integration would occur in the genome. Additionally, because the number of transposons integrated in the genome decreases over time, a large number of transposable elements are required (81). PB demonstrated the highest efficiency and stability in gene transfer (64, 82, 83). Even though SB insertional mutagenesis system is more random and less efficient, it can integrate transposons up to 10 kb in size (84), making it capable of delivering around 80% of human cDNAs (85). In contrast, the PB system can only insert cDNAs approximately 2.4 kb in size (86).

CRISPR/Cas9 transgenic glioblastoma mouse models

CRISPR/Cas9 is an RNA guided nuclease which is involved in prokaryotic immune systems (87–89). It has been used extensively to generate cancer models through genetic editing, providing a fast, inexpensive, and simple method to identify and study genetic determinants of cancer. CRISPR/Cas9 mouse models can be generated by injecting Cas9 mRNA with one or multiple single guide RNAs (sgRNA) directly into mouse somatic cells or germline embryos, which creates precise genomic edits at specific loci (Figure 4 A, B) (90). Depending on the type of DNA repair that took place, two kinds of mouse genome modifications will occur: constitutive knock-out tumor suppressor genes through non-homologous end joining and knock-in oncogenes through homologous recombination (91–93). The whole process takes around 2–3 months, which is much faster than the Cre-LoxP system.

Although the CRISPR/Cas9 system can be used in both somatic and germline cells, researchers are more likely to use it to edit somatic cells in transgenic or wild type mice. Plasmids targeting specific genes are first edited by the CRISPR/Cas9 system, then injected into germline transgenic mouse models generated from Cre-LoxP and other transgenic systems to create more accurate and precise knock-out or knock-in mouse models (44, 92). By injecting plasmids modified by the CRISPR/Cas9 system *in utero* at Embryo stage E13.5 days, researchers generated

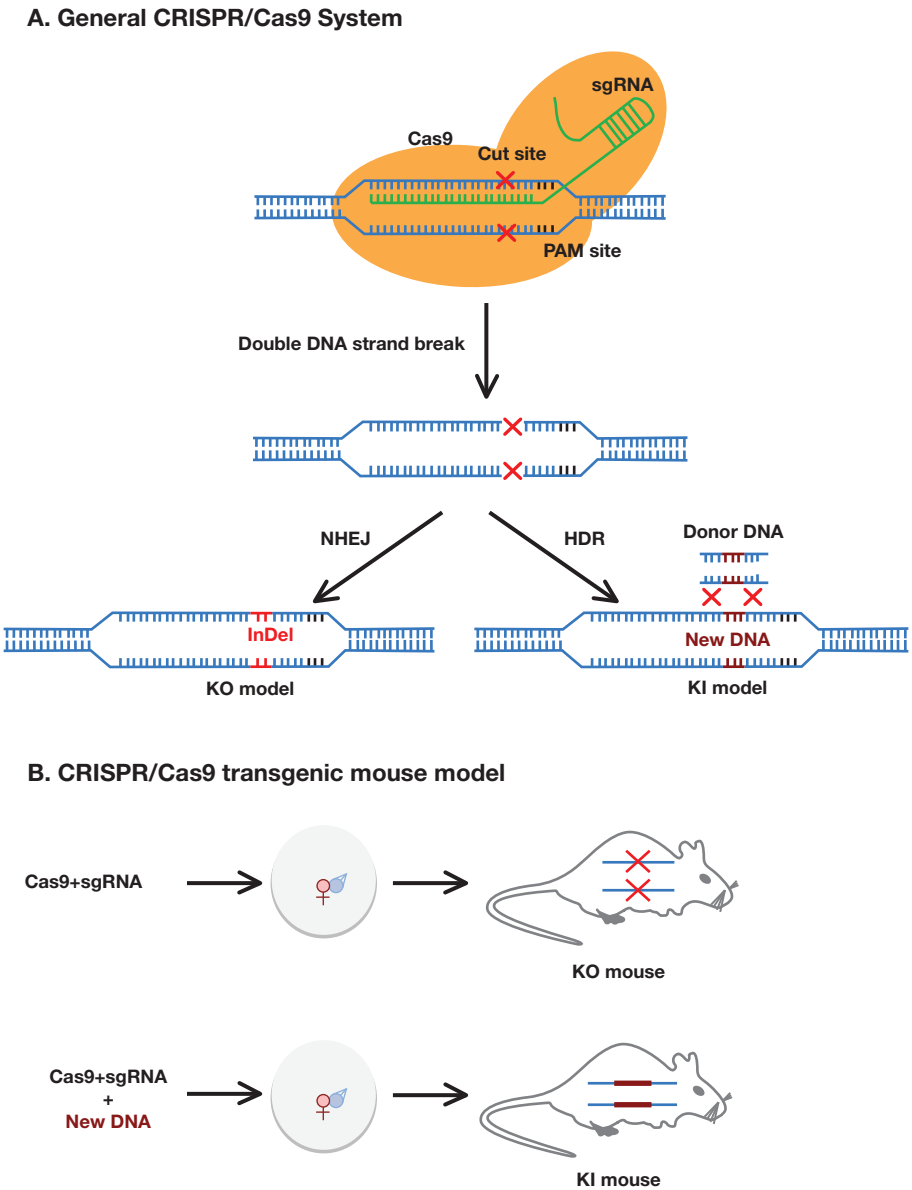

Figure 4. The CRISPR/Cas9 System. A. General CRISPR/Cas9 system; in the general CRISPR/Cas9 system, Cas9/sgRNA complex recognizes the complementary 20-nucleotide genomic sequence with a downstream protospacer-adjacent motif (PAM) sequence; it cuts three nucleotides upstream of the PAM sequence to induce double-strand DNA breaks (DSBs); the DSBs are then repaired through two major mechanisms: NHEJ pathway which is usually for knock-out genes and HDR pathway which is usually for knock- in genes. **B.** CRISPR/Cas9 transgenic mouse model; CRISPR/Cas9 germline transgenic mouse models are generated by injecting Cas9 mRNA with one or multiple single guide RNAs (sgRNA) directly into mouse germline embryos; two kinds of mouse genome modifications will occur: constitutive knock-out tumor suppressor genes through NHEJ and knock-in oncogenes through HDR.

Ptch1, p53 double loss mouse model, instead of using the traditional method of breeding *Ptch1*$^{+/-}$ mice with *p53*-null mice (92, 94). The highly aggressive glioma developed in a short period of time in all mice, and the tumors produced via CRISPR/Cas9 are mostly similar to tumors produced in germline transgenic mice (92).

With continued development, gene editing techniques are now used more often in combination for glioblastoma mouse models. Chen et al. combined the CRISPR/Cas9 system and *PB* transposase lineage labeling to induce somatic mutations in NPCs. They used *PB* transposase system producing GFP or RFP signal which can label the lineage of CRISPR-targeted progenitors *in vivo*. At the same time, they used CRISPR/Cas9 constructs containing sgRNAs targeting *NF1, PTEN*, and *p53*, alone or in combination to generate *NF1, PTEN,* and *p53* deletion in somatic cells. In this way, they demonstrated that CRISPR/Cas9 combined with *PB* transposase lineage labeling is a convenient way to produce unique tumors caused by somatic mutation in neural progenitors (44). The CRISPR/Cas9 system is a fast method to provide versatile gene editing, making it extremely useful. However, one major limitation of this nuclease technology is the non-specific and off-target cutting of DNA sequences. Because the Cas9 nuclease randomly cuts within the sequence and can target some slightly different sequences, some undesired mutations may occur, which could significantly affect the phenotype of the generated mouse models (95).

Viral vector delivery system glioblastoma mouse models

Viral vector delivery is another approach that can modify multiple genes to generate somatic transgenic mouse models (37, 96–98). Several types of virus vectors can be used to deliver transgenic or mutant genes, including adenovirus, adeno-associated virus, lentivirus, retrovirus, etc. The main difference between lentiviruses and retroviruses is that lentiviruses are capable of infecting non-dividing and actively dividing cell types, whereas retroviruses can only infect mitotically actively dividing cell types. This means lentiviruses can infect a greater variety of cell types than retroviruses (99). Combining the lentivirus transfection-induced model with targeted conditional knock-out/knock-in transgenic mouse models makes it more convenient to study the pathways that drive glioblastomas (90, 100, 101). Lentivirus engineered to co-express the TAM induced CreER^{T2} along with *PDGFB* and GFP protein can spatially and temporally control the deletion of the floxed genes in specific cells as well as easily track the transduced cells. The glioblastoma penetrance of this model was as high as 88.5% (102).

The most widely used retrovirus induction system is the RCAS-TVA delivery system (42, 102). This approach uses replication-competent avian sarcoma-leukosis retrovirus (RCAS) vectors to target cells that are engineered to express cell surface receptor TVA (a receptor for the avian leukosis viruses (ALV) envelope glycoprotein) (7). RCAS-TVA transgenic mouse models are created by injecting RCAS vectors directly into mouse brain that expresses the RCAS receptor TVA (90, 103, 104). Combining the RCAS-TVA system with Cre-loxP and other transgenic systems provides a versatile method for producing glioblastoma mouse models containing different types of proliferating cells targeting different tissues (105). To generate glioblastomas, viruses were injected into different locations in

the brains of wild type mice or mice with various deleted tumor suppressors to overexpress different types of oncogenes in *Nes* and *GFAP* positive cells (43, 97, 104). These studies provided a new way to quickly establish mouse models so that the therapeutic responses of gliomas can be simultaneously compared (97). Glioblastoma mouse models can also be generated by combining RCAS-TVA system with CRISPR-Cas9 transgenic gene editing system to somatically delete tumor suppressor genes *p53, Cdk2a*, and *PTEN* in neural stem cells (NSCs) *in vivo* (90). This RCAS/TVA/Cas9 system is extremely versatile and accurate for somatic gene editing *in vivo* (90), which can help identify the various tumor-inducing factors of different glioblastoma types. One limitation of the RCAS-TVA system is that it requires the specific TVA-transgenic mouse strains. In addition, RCAS vector has a 2.5Kb DNA insert restriction. Genes of larger size cannot be inserted into the RCAS vector (7).

Transgenic mouse models are useful for observing specific genetic alterations involved in glioblastoma initiation and progression, but it is still uncertain whether the gene changes involved in these models truly mirror the tumor progression events in human glioblastomas. Most of the time, transgenic mouse tumors have specific gene mutations in specific cell types such that those tumors are more uniform and cannot completely reflect the phenotypic heterogeneity of human glioblastomas. To accurately reflect the heterogeneity of glioblastomas, mouse models have been created by combining several techniques to generate multiple complex genetic edits. In addition, tumor heterogeneity can also be maintained via transplantation of tumor specimen into mouse models (44, 90, 92, 97, 106).

TRANSPLANT GLIOBLASTOMA MOUSE MODELS

Besides gene ablation mechanisms, immune "escape" mechanisms may also play an important role in glioblastoma development (107). Even if more is known about the gene mutations related to glioblastoma, effective treatment is still difficult due to the microenvironment which can include immune suppressive cells, such as brain microglial and macrophages (23, 108). The brain tumor cells can escape from the immune cell surveillance, which facilitates glioblastoma aggression and can potentially induce drug resistance. Thus, understanding the function of the immune system in the glioblastoma microenvironment is most important for developing immune therapy for glioblastomas. Transplant models provide a natural tumor growth environment and have good control over tumor site and size, making them highly reproducible and excellent for tumor immunology studies and preclinical immunotherapy studies.

Transplantation of tumor cells into mice can rapidly generate experimental glioblastoma model for studying tumor biology and examining therapeutic methods. Many types of biological materials can be transplanted into mice brain by intracranial implantation (31, 109) or subcutaneous injection (110) techniques. This includes engineered murine tumor cells,such as GL261; engineered virus vaccines; Cre-LoxP, TAM/Tet/Dox induced tumor cells; and cancer cells/tissues from primary patient tumors (PDX). The injection can be done at either embryo stage or post-neonatal stage to induce experimental glioblastoma (7, 109, 111–113). Adult immunocompetent mice fail to tolerate the human-specific tumor

microenvironment (TME) features, while embryonic (E12.5) mice can be engrafted. The embryonic stage injection induces experimental glioblastoma that invade the mouse brain and exhibit the complex intact TME with vasculature, astrocytes, and immune cell infiltration (111, 114).

There are two types of transplant models: the allograft transplant model, which involves implanting tumor cells from the same species, such as mouse GL261 cell lines implanted into mouse brain (30, 112, 115, 116); and the xenograft transplant model, which involves implanting tumor cells cultured from different species, such as human glioblastoma cell lines implanted into mouse brain (117, 118). There are two techniques for the transplantation: stereotactic intracranial injection and subcutaneous injection. Intracranial injection is a more preferable approach used because it directly introduces glioma cells into the brain, where the tumor can develop under the naturally occurring immune environment to model glioblastoma progression and infiltration. Subcutaneous implantation lacks these characteristics (119).

Allograft transplant mouse models

Allograft transplant mouse models are usually produced in immunocompetent mice, which offers the intact immune system and same tissue context, and thus avoid immune rejection. The cell lines used in allograft mouse model include GL261, GL26, CT-2A, P560, and 4C8. The GL261, GL26, and CT-2A cell lines were generated from carcinogens, including N-ethylnitrosourea and 20-methylcholanthrene induced into C57BL/6 mice. P560 was from spontaneous VM/Dk mouse models. And 4C8 was from B6D2F1 mouse models (120, 121). These cell lines have their own characteristic immune markers that make them suitable for different studies (Table 2) (122–127). Among these cell lines, GL261 is the most widely used for many immunotherapy and gene therapy studies (128, 129). This cell line shares several characteristics with human glioblastomas (129–132). Histologically, GL261 tumors show features of ependymoblastoma (130). Immunologically, GL261 expresses high levels of major histocompatibility complex class I (MHC I) as well as MHC II, B7–1, and B7–2, CD31, CXC chemokine receptor 4 (CXCR4) (129, 131). Genetically, GL261 shares many gene mutations with human glioblastomas, including *RAS* oncogene and *p53* tumor suppressor gene point mutations (129, 132). In general, when 1×10^5 GL261 tumor cells are injected into C57BL/6 mouse brain in 2–4 μl, around 70% of mice will develop glioblastomas and survive for about 3–4 weeks (30, 109, 112).

Allograft transplant models have been used to study the immune mechanism for radiation therapy, immune checkpoint therapy, vascular endothelial growth factor (VEGF) therapy and vaccine therapy. Whole brain radiation therapy (WBRT) is one of the therapies tested in the GL261 model. Although WBRT itself has minimal advantage in terms of survival, this approach up-regulates β2-microglobulin expression in GL261 glioblastomas *in vivo* and *in vitro*, thus increasing $CD8^+$ T cell mediated antitumor immune response. When WBRT is combined with vaccine treatment, the long-term survival increased 40–80% (116). Immune checkpoint anti-PD-1 immunotherapy with radiation is another treatment that showed promise when tested in the GL261 mouse model by inducing activation and expansion in cytotoxic $CD8^+$ T cells. It can also allow the body

TABLE 2 Summary of allograft transplant cell lines

Cell Line	Established year	Mouse Strain	Histology	I.C. Cell numbers	Tumor initial time(pid)	Immune Characteristics	Citations
CT-2A	1992	C57BL/6	Anaplastic astrocytoma	8×10^4	15–20	Express Cd133, Oct, Nestin	(122, 172)
GL26	1969	C57BL/6	Ependymoblastoma	2×10^4	31	Express MHC I, lack MHC II	(123, 124, 173)
GL261	1970	C57BL/6	Ependymoblastoma	1×10^5	24–25	Express vimentin, c-Myc. K-ras mutation, p53 mutation, High levels of MHC I. Less MHC II, B7-1, and B7-2,	(174–176)
P560	1980	VM	Anaplastic astrocytomas	1×10^4	26	Have MHC I, Lack MHC II	(127, 177, 178)
4C8		B6D2F1	Oligodendrocytic astrocytes	1×10^4	51	Express MHC I and II	(125, 126)

to maintain long-term immunologic memory (116, 133). VEGF mediates angiogenesis, and its expression is highly correlated with malignant glioblastoma grade (134). GL261 mouse models have been used to test anti-VEGF combined with vaccination immune therapy. The therapy could significantly delay tumor progression and extend survival period, providing a foundation for further evaluation of the effects of antiangiogenic therapy in the context of endogenous or vaccine-induced inflammatory responses (112).

In addition to wild type C57BL/6J mice, GL261 has also been transplanted into C57BL/6J background transgenic mice to further study the different factors or mutated genes involved in glioblastomas. The Cre-LoxP system has been used to specifically knock-out H-2K^{b} or H-2D^{b} in targeted dendritic cells and macrophages in glioblastoma mouse models to study the role of each cell type in the activation of CD8^{+} T cells in response to these central nervous system immunological challenges. The role of each cell type in generating the CD8^{+} T cell responses was different. MHC I H2-K^{b} or H-2D^{b} antigen presentation by dendritic cells and macrophages in these model systems is non-redundant (30, 31, 115).

Xenograft transplant glioblastoma mouse models

Even though mouse GL261 glioblastomas have characteristics highly similar to human glioblastomas, the model cannot replicate the human immune system. Some studies have also shown that the GL261 cell line has genetically drifted and accumulated mutations (135). To reflect the human glioblastoma immune microenvironment, the xenograft transplant mouse model has been established.

Xenograft models are generated by transplanting human glioblastoma cells lines or fresh tissue into immunocompromised mice to induce glioblastomas. Hence this is also called patient-derived xenograft (PDX). This model maintains the genetic and the histological features of the primary tumor from glioblastoma patients. The cell lines or fresh tissue from glioblastoma patients share some similar genetic changes, such as mutation of *p53* tumor suppressor gene and *PTEN* gene, loss of *p14^{Arf}* and *p16*, and overexpression of *AKT* due to *PI3K/AKT* pathway up-regulation (136, 137). However, different cell lines or tissues have significant differences in histopathological characteristics. This results in the histology of human glioblastomas being highly variable. Multiple cell lines are being used in xenograft model, such as SF-7761, glioblastoma12, Hs683, etc. (118, 138, 139). Because the culture conditions in serial generation affect tumor cell phenotype and heterogeneity (140–142), researchers tend to implant freshly isolated tumor cells or tissue fragments without culture or only culturing for a short time (34, 143). Injecting fresh human glioblastoma tumor specimen provides the most direct attempt to capture important features of human glioblastoma without any *in vitro* selection or contact with serum.

Xenograft transplants use immunocompromised mouse strains. The most popular strains are: nude mice, severe combined immunodeficient mice (SCID), non-obese diabetic mice (NOD), non-obese diabetic severe combined immunodeficiency (NOD/SCID), NOD/SCID/interleukin-2 receptor gamma chain (IL2Rγ)^{null} (NOG/NSG), NOD/SCID/Jak3(Janus kinase 3)^{null} (NOJ), and recombination-activating gene 2 knock-out serial mice (Rag2^{null}), BALB/c Rag-2^{null}/IL2Rγ^{null} (BRG), Rag-2^{null}/Jak3^{null} (BRJ), BALB/c Nude Rag-2/Jak3 (Nude R/J) (Table 3). These strains are deficient in different immune cells, and this incomplete immune

TABLE 3 Summary of frequently used immunocompromised mice

Mouse Strain	Full name	Immune Characteristics
Nude mice	Athymic nude mice	No thymus, No T cells
SCID	Severe Combined Immunodeficient Mice	No T cells, no B cells
SCID/Beige	Severe Combined Immunodeficient Mice/ Beige	No T cells, no B cells, severe reduced NK cells
NOD	Non-obese diabetic mice	Pancreatic no T cells, impaired NK cells, macrophages and dendritic cells
NOD/SCID	Non-obese diabetic Severe combined immunodeficiency	No T cells, no B cells, impaired NK cells, macrophages and dendritic cells
NOG/NSG	NOD/SCID/interleukin-2 receptor gamma chain(IL2Rγ)^{null}	No T cells, no B cells, no NK cells, impaired macrophages and dendritic cells
NOJ	NOD/SCID/Jak3(Janus kinase 3)^{null}	No T cells, no B cells, no NK cells, impaired macrophages and dendritic cells
BRG	BALB/c Rag-2^{null}/IL2Rγ^{null}	No T cells, no B cells, no NK cells
BRJ	Rag-2^{null}/Jak3^{null}	No T cells, no B cells, no NK cells
Nude R/J	BALB/c Nude Rag-2/Jak3	No T cells, no B cells, no NK cells

cell depletion affects the transplant success rate (144, 145). Recently, NSG mice have been used more for PDX research because this strain has depleted interleukin-2 (IL-2) receptor gamma. IL-2 receptor gamma signaling pathway is essential for many types of hematopoietic differentiation, so the absence of this receptor causes a dysfunction in innate immunity such as NK cells. These characteristics make NSG mice an effective model for xenograft transplant of primary tumor tissues or cells (117, 146).

The mechanism of the many preclinical treatments has been tested using this model. Several human glioblastoma cell lines including wild type *H3.3* cell lines (SF9402, SF9427, SF9012 and GBM43) and *H3.3*^{K27M} mutant cell lines (SF8628, SF7761) were transplanted into female athymic nude mice to analyze the effect of GSK J4 treatment for *H3.3*^{K27M} -mutant cell *in vivo* and *in vitro*. The results demonstrated that GSKJ4 could reverse *H3.3*^{K27M} demethylation to serve as a therapeutic strategy for lethal pediatric glioblastomas (118). Temozolomide (TMZ), which induces cell cycle arrest at G_2/M and eventually leads to apoptosis, is an agent for chemotherapy used to treat glioblastoma (147, 148). TMZ is effective for some GBM cell lines in PDX models, such as Hs683 and U87, but not for T98G and U373 (138, 149, 150). The results obtained across different cell lines, suggest variability in glioblastoma characteristics and their role in responsiveness to TMZ. The mechanism of the viable response is not clear. Some studies showed that the resistance of GBM cell lines to TMZ therapy may due to level of methylated enzyme O6-methylguanine-DNA methyltransferase (MGMT). High levels of

methylated MGMT promotor showed more response to TMZ (151). But Dr. Egana et al. was not able to demonstrate MGMT methylation could influence patient survival in Glioblastoma. While combining TMZ with bevacizumab, an antiangiogenic antibody targeting *VEGF*, increased the survival of glioblastoma mice (152).

Xenograft transplant mouse models can preserve the genetic and histological complexity of the primary glioblastomas, but this model differs from patient tumors in many ways. Immunocompromised mice xenograft models do not have an intact immune system and lack the human tumor microenvironment. In addition, a high rate of copy number variations occurs in serial *in vivo* passaged xenografts, and the murine stroma can also gradually take over (34, 153). Xenografts with tissue directly from patients may be better than xenografts with cells that have been expanded *in vitro* (154), but the differences of immune system between human and immunocompromised mice means that PDX models may not accurately reflect the biological nature of glioblastoma in patients, which is a disadvantage when it comes to preclinical drug studies and chemotherapeutic drug studies. Therefore, it is imperative to find a mouse model that can investigate human glioblastoma development and immunotherapy efficiency in human TME with intact immune system.

Humanized mouse models

To obtain mouse models with fully competent human immune systems, which enable researchers to examine the interaction between the tumor, immune system, and microbiome for patient preclinical therapy, humanized mice have been generated. These mice have been extensively used for discovering effective immunotherapeutic agents and their combinations (155). Several types of humanized mouse models have been generated, such as PDX human hematopoietic stem cells (HSCs) humanized mice and human microbiota-associated (HMA) humanized mice (156–158). To create PDX HSCs humanized mice, scientists inject human peripheral blood mononuclear cells (hu-PBMCs) or HSCs, or specific HSCs such as Hu-CD34$^+$ HSCs (hu-CD34$^+$) directly into immunodeficient mice after 50–250 cGy whole body irradiation (117, 156). The success of the humanization process is that mice have more than 25% human CD45$^+$ cells in their peripheral blood. Hu-PBMCs mice develop T cells and B cells. It is a model for research on compounds for T cell immune modulation and graft rejection. Hu-CD34$^+$ humanized mice develop almost all human stem cell lines, including T cells, monocytes, macrophages, mast cells, myeloid (SGM3) cells, NK cells (IL-15), and dendritic cells. It is a more advantageous *in vivo* model for long-term studies in the fields of human immune cell biology, immuno-oncology, and infectious disease. Jackson lab provides several types of mouse model for different study purposes. The most popular humanized immunocompromised mice strains are NOD. *Cg-Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV IL-3, CSF2, KITLG)1Eav/MloySzJ (NSG-SGM3), and NOD,B6.SCID *Il2rγ*$^{-/-}$ *Kit*$^{W41/W41}$ (NBSGW) mice (NBSGW) (117). NSG-SGM3 mice delete IL-2, but express human IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor. These factors enable the stable transplant of human HSCs for humanization (117, 159). NBSGW mice carry c-Kit mutation to support the transplantation of HSCs without irradiation because c-Kit plays a role in cell survival, proliferation, and differentiation (160). A humanized mouse can also be generated by a conditional knock-in/out of a

specific human gene or a piece of genomic sequence to precisely target a certain tissue, such as SRG-15 mice with knock-in human SIRPA and IL-15 to develop the innate lymphoid cell subsets and NK cells (161); MHC NOG-dKO mice with double knock-out MHC class I and II in NOG mice (162); and NSG-SGM3-BLT mice which involves implanting human fetal liver and thymus fragments as well as hematopoietic stem cells into immunocompromised NSG-SGM3 and NOD/SCID mice (163).

In addition to the PDX HSCs humanized mice, recently scientists also tried to generate HMA humanized mouse models since research has shown that the gut microbiome is linked to some immune-mediated and metabolic pathologies such as obesity, type 2 diabetes, and cancer (163–166). Recent studies found that balance of commensal microorganisms is important for cancer etiology and that gut microbiota can impact the treatment for cancers (167). Although mouse and human share 85% similar genomes, they have significant differences in gut microbiota composition. Around 85% of mouse gut bacteria are not found in human (168). Considering the relationship of gut microbiota composition and cancer development, scientists generated HMA models to avoid the impact on immune system by gut microbiota composition. HMA is established by using microbiota transplantation to transplant human fecal microbiota to germ-free mice (169).

Several studies have used the HMA model (164, 165). After successfully establishing the HMA mouse model, GL261 glioma cells were intracranially transplanted to set up glioblastoma HMA model for studying the response to anti-PD-1 or anti-PD-1 combined with TMZ treatments. The mice that survived longer have higher IFN-γ and higher $CD8^+$/Treg ratio than those that survived shorter. This difference in treatment response was due to the difference in the microbiomes from different patients (164).

Humanized mouse models by themselves or combined with transgenic mouse models highlight a new way to investigate the relationship between glioblastoma development, human immune system, and human microbiota system. It also provides a new platform to study the anticancer immune response for specific immunotherapeutic interventions. However, xenograft PDX humanized mouse models still have challenges due to host innate immune response in immunocompromised mice to the engraft of human cells/tissues, limited lifespan of the mice, incomplete human immune function, and poor lymphoid architecture (155, 170). The HMA humanized models also have many biological and technical problems. Whether the human donor microbiomes are successfully transplanted into germ-free mice and whether this model is reproducible still needs confirmation. The mucus properties of germ-free mice are different from conventional mice, which may not completely reflect the human response (169, 171). In addition, the transplant procedure may destroy tumor tissue architecture. Therefore, more research is needed to determine whether transplant mouse models are suitable for glioblastoma studies.

CONCLUSION

Mouse models are extremely useful for studying the biology of glioblastoma. Scientists use mouse models suitable for their experiments to gain insight into mechanisms and factors concerning tumor molecular processes, tumor

progression microenvironment, and immune and preclinical therapeutics. Spontaneously induced tumors better reflect the natural tumor growth and immune environment change; transgenic mouse systems focus on the targeted genes and pathways for tumor progression; and transplant models are better for tumor immune therapy studies. From Cre-LoxP germline transgenic mouse models to virus vector transgenic somatic transgenic mouse models, many cutting-edge technologies are combined to create combinations of gene mutations that reflect the complexity of glioblastoma in human. This will help in identifying more genotype-specific susceptibilities of human glioblastoma types, manipulating the human glioblastoma epigenome, developing glioblastoma gene therapy and immune therapy in humans, and eventually enabling more personalized, genotype, and phenotype-based treatments for glioblastoma patients in the future.

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3

Cancer Stem Cells in Pediatric Brain Tumors

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Abstract: Cancer stem cells are a subpopulation of tumor cells that have the ability to self-renew, initiate tumors in model systems, and differentiate into non-cancer stem cells. They are also resistant to current standard of care treatments, such as radiotherapy and chemotherapy. Due to these properties, cancer stem cells contribute to tumor progression and recurrence and need to be inclusively targeted with therapeutic paradigms used in the clinical setting. This chapter covers the most up-to-date published information on cancer stem cells in the context of pediatric brain tumors. The characteristics of pediatric brain tumor cancer stem cells, including resistance mechanisms and differential genetic regulation that allow for the stem like phenotype, are presented. The current research on cancer stem cells in medulloblastoma, ependymoma, diffuse intrinsic pontine glioma, and pediatric gliomas as well as potential approaches that are being developed to target cancer stem cells are highlighted. Challenges in targeting cancer stem cells in the pediatric patient population are also discussed.

Keywords: cancer stem cells; chemotherapy resistance; pediatric neuro-oncology; radio-resistance; tumor initiating cells

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INTRODUCTION

There are more than 28,000 children in the United States currently living with brain tumors (1). Pediatric brain tumors are the second most common cancer in children and the leading cause of cancer related deaths (1, 2). There are many different types of pediatric brain tumors, and they have varying treatment options and survival outcomes. Advances in successful treatments and surgical techniques have allowed tumors, such as medulloblastoma and low-grade gliomas, to achieve a 5-year survival of about 75% (3, 4). Meanwhile, survival of other pediatric brain tumors, such as diffuse intrinsic pontine glioma (DIPG), high-grade glioma, and ependymoma remain dismal. DIPG, also known as diffuse midline glioma, is one of the most deadly pediatric brain tumors with an overall survival of 30% a year after diagnosis, and less than 1% after 5 years (5). Current treatments for pediatric brain tumors generally include surgery, radiotherapy, and/or chemotherapy (4). Even with these treatments, many pediatric tumors still remain incurable. Tumors like ependymoma have poor prognosis due to high rates of recurrence, adding another layer of complexity to treatment planning for pediatric tumors.

One of the reasons these pediatric brain tumors are difficult to cure and commonly recur is the existence of innate intra-tumor heterogeneity. Using genomic sequencing and clustering algorithms, brain tumors have been shown to be heterogeneous in nature and to have distinct subpopulations existing within the same tumor. One leading theory as to how this high level of heterogeneity connects to a high recurrence rate and low survival is called the cancer stem cell (CSC) hypothesis (6–8).

The CSC hypothesis is based around the existence of a subpopulation of cells within a tumor that are able to initiate new tumors in *in vivo* model systems. These cells can both self-renew and differentiate as a means to repopulate tumors by producing both more CSCs as well as differentiated non-CSCs (7, 8). CSCs can be rare, in some cases making up less than 3% of the cell population. However, if missed during surgery, the CSCs are capable of re-forming tumors, which results in tumor recurrence (7). CSCs found in brain tumors have also been shown to be resistant to aggressive radiotherapy and largely unaffected by standard chemotherapies, often leading them to be left behind after treatment (9). It has been shown CSCs will re-populate tumors and lead to resistant, aggressive secondary tumors (Figure 1) (9). Due to these aspects of CSCs, there exists a desperate need to not only further study this subpopulation in the context of pediatric brain tumors, but also to find therapeutics that target these cells to reduce recurrence.

In this chapter, we discuss the properties and features that make CSCs such a dynamic and important population to study, set in the context of their role in pediatric brain tumors. We first cover defining characteristics of CSCs, such as tumor initiation, self-renewal, and chemotherapy/radiation-resistant properties. Then we present different types of pediatric brain tumors that have known CSC subpopulations, and what work has been done to understand them. Lastly, we explore different ways researchers are targeting pediatric brain tumor CSCs.

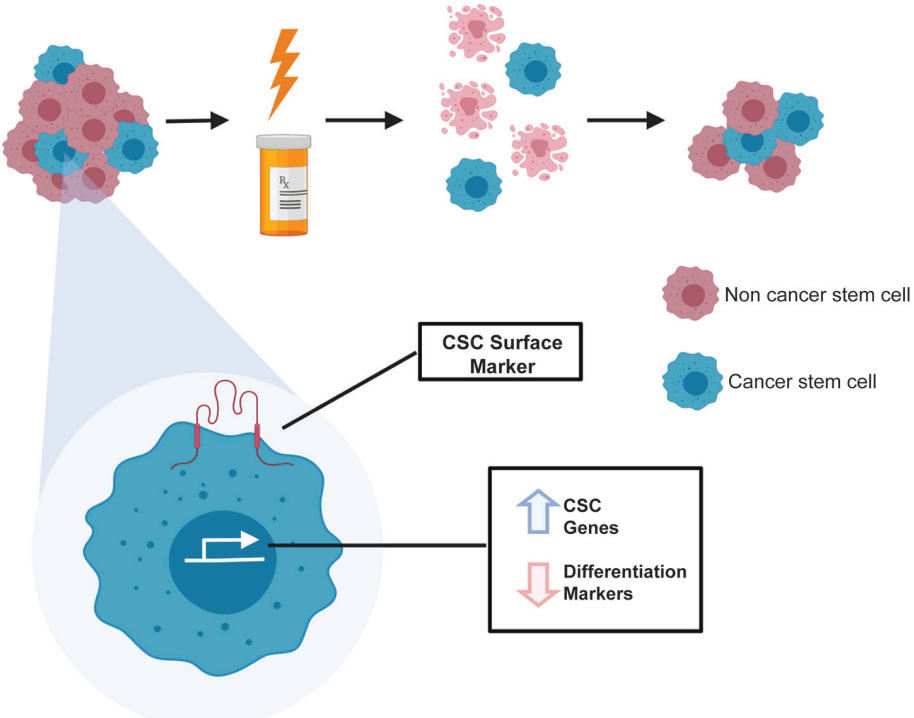

Figure 1. Pediatric brain tumor CSC phenotypes and genotypes. Pediatric brain tumors are heterogeneous in nature, containing both CSCs and non-CSCs. When bulk tumors are treated, with either radiotherapy or chemotherapies, the radio- and chemo-resistant CSC subpopulation remains and can repopulate the tumor. CSCs can be sorted from bulk tumors by cell surface markers, such as CD133. CSCs have intrinsic characteristics such as gene regulation that keep them in a progenitor/stem state via upregulation of stem genes and downregulation of genes related to a more differentiated cell state. These changes give CSCs the ability to self-renew and initiate tumor formation, thereby contributing to tumor recurrence. Differential gene regulation from non-CSCs is also thought to contribute to CSC therapeutic resistance, with DNA damage repair genes and drug efflux transporters upregulated, while pro-apoptotic pathways are downregulated. Created with BioRender.com.

PEDIATRIC BRAIN TUMOR CSC PROPERTIES

CSCs were first identified in acute myeloid leukemia (AML), where researchers discovered a subpopulation of tumor cells that were able to proliferate, differentiate, and self-renew, as shown by serial transplantation in mouse models (10, 11). Later, CSCs were found in numerous solid tumors, such as breast, prostate, colon, lung, and brain (12–17). CSCs have been identified in a variety of brain tumors, both adult and pediatric, such as glioblastoma, ependymoma, and medulloblastoma. Common practice for isolating CSCs is to first dissociate a bulk tumor into a single cell suspension, sort for the CSCs via reported cell-surface markers using fluorescence-activated cell sorting (FACS) or magnetic sorting, and then

functionally validate the self-renewal capabilities, as well as the ability to accurately recapitulate the tumor of origin in orthotopic xenotransplantation studies in mice (9). The most common CSC cell-surface marker used for brain tumors is the extracellular glycosylated antigen known as CD133 (18). Validation of the CSC phenotype can be done through *in vitro* limiting dilution assays, in which lower and lower cell numbers are plated per well of a tissue culture plate down to one cell per well. The cells are given time to expand with the final metric evaluated being the presence or absence of a tumorsphere in each well. This can be quantified using the eLDA calculator, which is an open access online algorithm that calculates the frequency of CSCs present in a sample (19). Most important for functional validation, however, is an *in vivo* orthotopic limiting dilution assay where CSCs and matched non-CSCs are implanted intracranially in lower and lower cell numbers and the ability to initiate tumor formation is evaluated. CSCs alone are able to form a tumor that recapitulates the characteristics of the original patient tumor whereas the non-CSCs are unable to form tumors in mouse models. This ability to initiate tumors is a hallmark of functionally defining CSCs. Ependymoma, medulloblastoma, pediatric glioblastoma, and DIPG are some of the pediatric brain tumors in which CSC populations have been isolated and functionally validated via the *in vivo* functional assay (20–25).

CSC treatment resistance

As well as being able to re-form tumors *in vivo,* CSCs have also been shown to be resistant to irradiation and chemotherapy in many different tumor types. This evidence further supports the hypothesis that these cells contribute to tumor recurrence, since resistant CSCs can persist after administration of conventional treatments. In fact, many therapeutics have shown efficacy for non-CSCs, while having little to no efficacy for CSCs (9). In certain brain tumors, CSCs have been shown to be resistant to both radiation and chemotherapy due to their inherent cellular properties. One example is glioblastoma CSCs having an enhanced DNA damage response (9, 26–28). This means that when DNA in glioblastoma CSCs is damaged by either radiation or chemotherapy, they are better able to repair the damage and survive than non-CSCs that die as a result of the increased DNA damage. It has been shown that CSCs in adult gliomas have an increased DNA damage repair capability via the ATR/Chk1 and ATM/Chk2 pathways, enhanced reactive oxygen species (ROS) scavenging, which protects against damage induced by radiation, and activation of cell survival pathways like PI3K/AKT (26, 29, 30). For pediatric gliomas, ependymoma, and medulloblastoma, CSCs have been shown to be resistant to many DNA damaging chemotherapeutics. This increased DNA damage response (DDR) is thought to be due to many factors discussed above, as well as other molecular aspects such as downregulation of apoptotic pathways, upregulation of anti-apoptotic pathways, and upregulation of pro-survival pathways (22). In general, CSCs are also thought to achieve resistance through slower replication rates, increased drug efflux, alterations of cell death pathways, and alterations in drug metabolism (31).

An example of upregulated drug efflux pumps in CSCs is the ABC transporter family, which has been attributed to CSC resistance mechanisms. ABC transporters have been shown to play a role in drug resistance in many cancer types, due

to their ability to efflux cytotoxic drugs and maintain drug concentrations inside cells at sub-therapeutic levels (29, 32). Temozolomide and mitoxantrone are some of many drugs glioma CSCs have been shown to be resistant to, partly due to ABC transporters (33). In fact, temozolomide has been shown to increase the fraction of adult glioma CSCs in treated tumorspheres (33). ABC transporters are thought to be able to pump the chemotherapies out of the CSCs, leading to cell survival. ABC transporters have been found to be overexpressed in CSCs, and their presence correlates with high levels of drug resistance (29, 33, 34).

Another potential resistance mechanism described in brain tumor CSCs is increased aldehyde dehydrogenase (ALDH) activity. ALDH proteins play a role in the maintenance of CSCs and have been shown to be a marker of radio-resistance in gliomas. These also correlate with clonogenicity, or self-renewal ability, as well as tumor initiation *in vivo* (29). The ALDH1 and ALDH3A1 proteins are thought to play a role in resistance to radiation through the reduction of free radicals in the cells caused by radiation therapy, through the production of NAD(P)H (29).

CSC genetic changes in pediatric brain tumors

CSCs have been shown to have many genetic and phenotypic differences when compared to non-CSCs. Investigations are ongoing to understand the overall genetic makeup of CSCs, such as the changes in CSCs that allow for increased resistance to conventional treatments. While a majority of what we know about genetic and epigenetic changes in CSCs comes from adult tumor research, this has been explored in some pediatric brain tumors as well. CSCs overexpress a group of genes that allow them to exhibit stem cell characteristics. One example is medulloblastoma in which several molecular signaling pathways are upregulated in CSCs, including Sonic Hedgehog (SHH), MYC proto-oncogene, and Notch. SHH is a pathway that promotes progenitor proliferation, and over-activation is linked with tumorigenesis (22). Indeed, SHH target genes, *Gli1* and *CyclinD1I,* are overexpressed in medulloblastoma CSCs. CSC tumorsphere self-renewal capabilities were also shown to be dependent on N-MYC, and were associated with medulloblastoma CSC stemness (22). Notch signaling was found to be important in the regulation of CSCs by maintaining them in an undifferentiated state (22). For example, DIPG exhibits amplification of *ERBB1* and mutations in the *TP53* gene. In childhood gliomas, mutations in *H2F2A* and *DAZZ* were found. Current work is being performed to uncover how these genetic changes promote CSCs stemness in their respective tumors (9). In ependymoma, CSCs have been found to have gene expression profiles very similar to radial glia cells (RGCs), a type of progenitor cell found in the wall of neural tubes during the earliest stages of development, which play an important role in neuronal fate (20, 35). *CD133, Nestin, RC2* and *BLBP* were all shown to be overexpressed in ependymoma CSCs as well as RGCs (20). Ependymoma CSCs grown in a subcutaneous xenograft model also show a high degree of similarity with adult neural stem cells (NSCs) (21). Messenger RNA (mRNA) expression for genes such as *EPHB-EPHRIN* and *Notch* are similar between the two and both play a role in maintaining stem cell states (20, 21). This highlights a key property of brain tumor CSCs: they genetically have significant overlap with progenitors and stem cells native to the brain. This is true for both pediatric and adult brain tumor CSCs, as adult glioblastoma CSCs harbor transcriptional programs akin to progenitors as well (36, 37).

In summary, pediatric and adult CSCs harbor genetic upregulation of stem cell genes, allowing them to stay in a pluripotent state. Resistance has been linked to this stem fate, so research has been done in adult glioblastoma CSCs to push the stem cells to a more differentiated, and therefore more sensitive fate (38, 39). PARP inhibitors, high-Z metal nanoparticles, PI3K inhibitors, DNA repair inhibitors, pimozide, CBL0137, various microRNAs, and many other therapeutic techniques have been shown to sensitize glioblastoma to irradiation (39–43). PARP inhibitors have been shown in glioblastoma to decrease the CSC frequency *in vivo*, leading to a higher percentage of non-CSCs in the tumor. They also led to a significant increase overall survival in murine models when PARP inhibitors were combined with radiotherapy, compared to either therapy alone (40). CBL0137, an anticancer drug that targets the FACT complex, has been shown to increase asymmetric cell division in glioblastoma CSCs, resulting in an increase in non-CSCs (38). The inhibition of FACT in glioblastoma CSCs resulted in downregulation of *SOX2*, *OCT4*, *NANO*, *OLIG2*, and *NES* on the mRNA level (38). Together, these data highlight the importance of understanding the genetic drivers behind CSCs and potential ways researchers can develop therapeutics to target these genetic differences.

CSCS ACROSS PEDIATRIC BRAIN TUMOR TYPES

There are many overlapping characteristics between all pediatric brain tumor CSCs, such as tumor initiation, increased resistance mechanisms, as well as stem genes upregulated, resulting in an undifferentiated phenotype. Despite the similarities, medulloblastoma, ependymoma, DIPG, and gliomas all contain genetically distinct CSC subpopulations, specific to each tumor type (Table 1). The following sections explore what is known about the CSC population in each of these cancers.

Medulloblastoma CSCs

Medulloblastoma is the most common pediatric brain tumor. Medulloblastoma was shown to have intratumor heterogeneity, aiding the discovery of CSCs with unlimited self-renewal, increased invasion and motility, and thus the ability to contribute to tumor recurrence (22). CD133-positive medulloblastoma tumor cells have been shown to be able to self-renew *in vitro* (22). *In vivo,* when as little as 100 CD133-positive cells were intracranially injected into an immunodeficient mouse, the resultant tumor resembled the original medulloblastoma patient tumor, whereas CD133-negative cells, regardless of number injected, did not form a tumor (22, 44). It has also been shown that Nestin overexpressing progenitor-like cells in transgenic mouse models develop gliomas and medulloblastomas, showing Nestin as another CSC marker in medulloblastoma (45). Separately, it has been shown that patients with tumors displaying prominent CSC signatures correlate with poor prognosis (22).

Medulloblastoma has been described to have four subtypes, WNT, SHH, Group 3 and Group 4, with the latter two being the most aggressive (46–49). CSCs have been identified in Group 3, and were demonstrated to be able to form

TABLE 1 Summary of published findings on CSCs in pediatric brain tumors

Tumor Type	Description	Markers and Genes	Example Therapeutics Targeting CSCs	References
Medulloblastoma	Increased invasion and motility, high recurrence, 100 CSCs enough to form tumor in mice, self-renewal	CD133 positive CD15 positive Sox2 positive high Nestin expression high MYC expression	Smo-agonist (to inhibit SHH pathway), vismodegib, PI3K/Akt inhibitors	22, 44, 45, 50
Ependymoma	Tumor initiation, self-renewal, resistance to conventional chemotherapies	CD133 positive high Nestin expression RC2 positive	Temozolomide, VP16, vincristine, cisplatin, vorinostat	20, 21, 51
DIPG	Self-renewal, tumor initiation, small fraction of cells within the tumor	Ki-67 positive Olig2 positive Nestin positive GFAP positive PDGFRα positive		23, 24, 52, 53
Glioma	Resistant to radiation, resistant to chemotherapy, tumor initiation, leads to recurrence, pediatric glioblastoma was able to recapitulate tumor (antigenic with hemorrhagic areas, highly proliferative, highly vasculature, necrotic core)	CD133 positive High VEGF expression	Oncolytic herpesvirus, anti-VEGF, bevacizumab	25, 54, 55

neurospheres in culture, with upregulation of *CD133*, *Nestin*, and *Musashi*, which are all stem cell gene markers (50). These medulloblastoma CSCs were able to propagate tumor formation in mice, and able to recapitulate the primary tumor (50). Group 3 medulloblastoma also includes MYC amplification and overexpression, which is essential for medulloblastoma stem cell initiation and self-renewal properties, and correlates with the aggressiveness of the stem cells in these tumors (22). These cells also display therapeutic resistance in the form of quiescence, regulation of key pathways (downregulation of apoptotic pathways and upregulation of pro-survival pathways), and manipulation of microenvironmental factors (hypoxia) (22, 50). Additional research is needed to investigate the other subtypes and any CSCs they main contain.

Ependymoma CSCs

Ependymoma tumors are found primarily in children but can occur in patients of all ages. Ependymomas are generally found in the spine in adult patients and have a good prognosis, while pediatric patients tend to have intracranial ependymomas that correlate with poor outcomes. A distinct CSC subpopulation has been identified in intracranial ependymomas. These ependymoma CSCs have also been shown to be capable of tumor initiation, and are also resistant to conventional chemotherapies (21). Ependymoma CSCs have been able to recapitulate the original tumor when orthotopically implanted into a mouse (20, 51). Several studies have been done in order to target these resistant populations. The anticancer drugs temozolomide (alkylating agent), VP16 (topoisomerase II inhibitor), vincristine (vinca alkaloid), cisplatin (alkylating agent), and vorinostat (an HDAC inhibitor) have all been used to target ependymoma CSCs with varying degrees of success (21, 51). In some studies, temozolomide and vorinostat both decreased ependymoma CSC viability, while another study has shown ependymoma CSCs are resistant to temozolomide, VP16, vincristine, and cisplatin (21, 51). Thus, a reliable way to target these CSCs remains elusive.

DIPG CSCs

DIPG is an incurable pediatric brain tumor with less than a 1% 5-year overall survival rate and has one of the worse prognosis of any pediatric brain tumor with a mean age of 6–7 years old at diagnosis (52). DIPG CSCs have not been studied in depth, but it has been found that primary cell lines from DIPG patients had a population of CSCs that could self-renew *in vitro* and form tumors in immunodeficient mice *in vivo* (23, 53). In another study, DIPG was shown to contain a subpopulation of cells highly expressing stem cell genes (24). DIPG CSCs are a genetically distinct subpopulation defined by single cell RNA-seq and were found to also be genetically distinct from other glioma CSCs (24). DIGP tumor cells grown in stem promoting media have tumor-initiation capacity. Although not quantified, it was demonstrated that the CSCs in DIPG were a minority, only making up a small fraction of the total tumor. The total tumor population had a differentiated signature, similar to that described within the CSC hypothesis (24).

Gliomas CSCs and other pediatric brain tumors

High-grade gliomas (HGG), which include pediatric glioblastoma, represent about 10% of pediatric brain tumors. Pediatric glioblastoma has high morbidity and mortality and about a 20% overall survival rate, and this outcome can be in part attributed to glioma CSCs (25). The CSCs have been shown to be resistant to radiation and chemotherapy and to have the ability to repopulate the tumor, causing recurrence after initial treatment in *in vivo* model systems (25). Glioma CSCs have also been shown to able to accurately recreate HGG tumors in these model systems, in terms of epigenetic post-translational modifications, copy number alterations, and DNA mutations (54). The CSCs also formed neurospheres *in vitro*, validating self-renewal properties (54).

In one specific example, a pediatric glioblastoma from a 4 year old patient was sorted for CD133-positive cells which were then injected orthotopically into immunocompromised mice (55). The CSCs formed tumors that displayed highly antigenic and hemorrhagic areas and had areas that were highly proliferative, with increased vasculature and necrosis (55). It was identified that pediatric glioblastoma CSCs had elevated levels of vascular endothelial growth factor (VEGF), a signaling protein known for its role in angiogenesis (55). The level of VEGF was 10- to 20-fold higher in CSCs compared to the non-CSCs, highlighting a potential way to target the CSCs *in vivo* (55).

TARGETING PEDIATRIC CSC

Targeting pediatric CSCs will be pivotal for eradicating tumors completely and reducing recurrence. Medulloblastoma CSCs have been targeted in a number of ways. Inhibiting Notch signaling has been shown to reduce CD133-positive cell count almost 5-fold, while also increasing apoptotic rates 10-fold (9). Inhibition of the SHH pathway via Smo-antagonist reduced Nanog expression and inhibited the self-renewal ability of the medulloblastoma CSCs (22). The SHH pathway has also been shown to play a key role in medulloblastoma CSC self-renewal and maintenance via Bmi-1 promotion of CSC tumorigenicity (22). However, targeting this pathway with vismodegib led to increased CSC quiescence instead of leading to cell death (22). Targeting another pathway, PI3K/Akt in a mouse model of medulloblastoma, led to radio-sensitization of CSCs and an increase in apoptosis (22, 33).

As mentioned earlier, ependymoma CSCs have been shown to be sensitive to vorinostat, and to some degree to temozolomide. It was shown that vorinostat decreases tumorsphere-initiating capacity and induced differentiation. Ability to initiate tumors, or form tumorspheres *in vitro*, is a hallmark of CSCs, so the ability of vorinostat to stop the formation of these spheres shows potential for targeting ependymoma CSCs (21). Temozolomide was shown to have no effect on ependymoma CSCs, however in another study, temozolomide was found to decrease tumor initiation, moderately increase survival *in vivo* in an intracranial mouse model, and decrease subcutaneous tumor volume in combination with VP16 (21, 51). Much more remains to be done to find a reliable treatment for pediatric CSCs.

There have been many creative ways developed to target CSCs. One proposed approach is via an oncolytic herpesvirus, which has been used to target HGG CSCs. The premise is to target and kill the CSCs and non-CSCs, while sparing normal brain tissue (25). Pre-clinical studies have shown engineered oncolytic herpes simplex virus can infiltrate, replicate within and then lyse HGG CSCs, leading to prolonged survival in *in vivo* mouse models intracranially injected with gliomas (25). Retinoic acid has also been shown to differentiate glioma CSCs. However, this has not been uniformly seen in all gliomas (56). Bevacizumab, an anti-angiogenic drug targeting VEGF, has been used to treat pediatric glioblastoma CSCs and has demonstrated suppressed growth in xenograft models (55). Bevacizumab is used now in combination with the current standard of care, temozolomide after surgery and radiotherapy, in clinical trials (ACNS0822) (25).

Current challenges in treating pediatric brain tumors

One of the most complicated factors in treating pediatric brain tumors is preserving the developing brain while simultaneously eradicating the malignant tumor cells. Since CSCs and normal neural stem cells (NSCs) and neural progenitor cells (NPCs) have many overlapping characteristics, phenotypes, and genetic expression, there is potential to damage the normal developing brain tissue during tumor treatment (11). Therapeutics need to be developed with this in mind, in a way that targets CSCs and not normal NSCs/NPCs which are necessary and critical to the developing brain. Treatments for pediatric neuro-oncology in general must be particularly non-toxic to the vulnerable young brain, with a number of therapies potentially leading to irreversible damage, loss of cognition, and stunting brain development. Targeting tumorigenic pathways, specific growth factors, and microenvironmental factors that play a role in CSC plasticity are the suggested ways of specifically targeting CSCs (9).

Current treatment for pediatric brain tumors is surgery, irradiation, and/or chemotherapy. Chemotherapy is occasionally used in very young children in an attempt to stall tumor growth until they are of an age where they can tolerate more aggressive treatments like radiotherapy. However, chemotherapy also has drawbacks, some of which include early growth failure, nausea, cachexia, and deficiency in nutritional uptake (57). These effects are long term, as even replacement hormonal therapies cannot completely compensate for growth issues (58–62). Neurological defects leading to long term health issues, including major visual defects, severe hearing loss, and trouble with schooling, have been shown in patients given radiation treatment when less than 4 years old, (63, 64). One study found that only 1 in 3 long-term survivors of pediatric medulloblastoma were able to live completely normal lives, with the other 66% unable to be employed and reliant on social support network (63). These effects are thought to be a direct result of irradiation of the developing brain. Recent advances in radiotherapy, such as proton therapy, offer much better options for minimizing collateral damage to the brain. However, there is still a desperate need for new therapeutic interventions that would either allow for the postponement or replacement of irradiation in treating pediatric brain tumors, and specific targeting of CSCs that would not affect NSCs/NPCs.

While these are daunting hurdles, progress is being made to better treat pediatric patients. Firstly, understanding the biology and research from basic science to clinical trials is underway in order to create better treatments. Multidisciplinary collaborations between researchers aim to improve patient treatments via the better design of clinical trials (65, 66). Another step forward is identifying pediatric patients whose tumor allows for less intensive treatment. If less chemotherapy and/or radiation can still be used to cure the tumor, this will result in lessening of unnecessary side effects (66). Medulloblastoma is an example of these concepts. Basic science research studying medulloblastoma identified the WNT subtype and correlated it was a positive prognostic outcome. There are ongoing clinical trials for reducing radiotherapy in patients with the WNT subtype (66).

CONCLUSION

Pediatric brain tumors are a serious, life altering occurrence in which multiple factors need to be balanced for optimal treatment, including protecting the developing brain, while still aggressively targeting the malignant tumor cells. Pediatric brain tumors have been shown to have intra-tumor cell heterogeneity, with a small population of CSCs present. So far, CSCs have been identified in gliomas, DIPG, ependymoma, and medulloblastoma. These cells have been shown to be capable of self-renewal and tumor initiation, two major hallmarks of CSCs. CSCs are also resistant to radiotherapy and many different chemotherapies, leading to the hypothesis that they play a key role in tumor recurrence and ultimately contribute to the high morbidity of these tumors. New ways of treating these tumors that inclusively target the CSC subpopulation are essential. Work has been done to specifically eradicate these CSCs therapeutically by inhibiting key pathways or targeting unique characteristics. Moving forward, monotherapies or combination treatments that treat all subpopulations of cells within the tumors, including CSCs, will result in prolonged survival and overall better outcomes for patients.

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4

Emerging Roles of Wild-type and Mutant IDH1 in Growth, Metabolism and Therapeutics of Glioma

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Abstract: Glioblastoma is one of the most devastating human malignancies and is categorized into primary and secondary glioblastoma subtypes that develop through different genetic pathways. Isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2) are key enzymes linking cellular metabolism to epigenetic regulation and redox states. Hot spot mutations of *IDH1* is early and frequent genetic alterations in secondary glioblastoma as well as in grade II and III glioma and represent a major biomarker with diagnostic, prognostic, and predictive implications. Mutant IDH proteins acquire neomorphic enzymatic activity to produce D-2-hydroxyglutarate, a putative oncometabolite that could induce epigenetic changes

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at DNA and RNA levels. On the other hand, recent studies show that primary glioblastoma increases expression of wild-type IDH1, which confers therapeutic resistance. In this chapter, we introduce the current understanding of the biological roles of wild-type and mutant IDH enzymes in glioblastoma. We discuss the challenges hampering the development of IDH targeted therapeutics and the current status of IDH1 mutant inhibitor development.

Keywords: glioblastoma; glioma; IDH1; metabolism; therapeutics

INTRODUCTION

Gliomas are the most common primary malignant brain tumor in adults. Many WHO grade I gliomas are well-circumscribed, surgically curable tumors and have different molecular drivers than those seen in grade II, III, and IV gliomas. Even though grade II glioma is categorized as a low-grade glioma, it is incurable due to its diffusely infiltrative nature and that it almost inevitably progress to high-grade III glioma and grade IV secondary glioblastoma over time (1). Remarkably, over 70% of grade II gliomas and secondary glioblastoma possess heterozygous missense mutations in the gene encoding cytosolic enzyme *IDH1* (2–4), which confer a neomorphic enzyme activity that converts α-ketoglutarate (αKG) to D-2-hydroxyglutarate (D-2HG) (Figure 1) (5). Mutations in mitochondrial isozyme IDH2 have also been identified in gliomas, but they are much less common and mutually exclusive with mutations in *IDH1* (3, 6, 7). Though naturally existing D-2HG is at negligible levels, the intracellular concentrations of D-2HG reach 10–30 mM in the glioma with the *IDH1* mutation (5). D-2HG appears to be a major intracellular effector of *IDH1* mutated glioma and is considered as an oncometabolite, altering epigenetics and setting the cellular state permissive to malignant transformation (8–10).

There are three distinct groups of gliomas with different molecular drivers, mutations, epigenetic signatures, and clinical behavior: (i) *IDH* wild-type gliomas (primary glioblastoma); (ii) *IDH* mutant with a 1p/19q deletion; and (iii) *IDH* mutant with a p53 mutation (11). This distinction between the three groups of

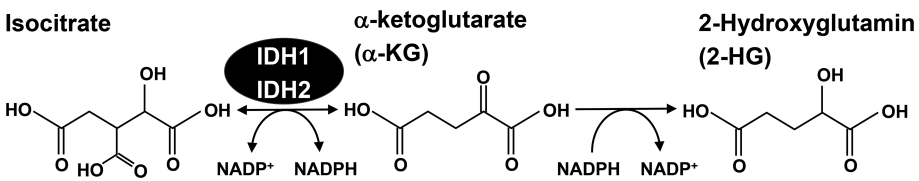


Figure 1. Wild-type IDH converts isocitrate and α-ketoglutarate to each other, and mutant IDH converts α-ketoglutarate to 2-hydroxyglutarate. Isocitrate dehydrogenase (IDH) interconverts isocitrate and α-ketoglutarate. Three subtypes of human IDH are known: IDH1 (cytosolic, $NADP^+$ dependent) and IDH2 (mitochondrial, $NADP^+$ dependent) and IDH3 (mitochondrial, NAD^+ dependent). IDH1 and IDH2 mutations have been reported in a variety of cancers such as, glioma, acute myeloid leukemia and bile duct cancer. Mutated IDH converts α-ketoglutarate to 2-hydroxyglutarate, which worsens the prognosis of gliomas. IHD1 and IDH2 mutations produce D-2-hydroxyglutarate, which has been considered "oncometabolite."

gliomas is currently driving clinical management as well as placing greater emphasis on the molecular and genetic differences of *IDH* mutant and *IDH* wild-type gliomas. This chapter discusses the role of wild-type and mutant IDH1 enzymes in the progression of glioma, and emerging therapy targeting the glioma with wild-type or mutant IDH1.

METABOLIC CHARACTERIZATION OF WILD-TYPE IDH1 GLIOMA

There are three isocitrate dehydrogenase isozymes—IDH1, IDH2, and IDH3—that are expressed in mammalian cells. IDH1 is a cytosolic enzyme, while IDH2 and IDH3 are mitochondrial enzymes. Both IDH1 and IDH2 use $NADP^+$ as an electron acceptor to convert isocitrate to αKG, co-producing an NADPH per reaction. IDH3 uses NAD^+ as an electron acceptor. Notably, the ratios of NADPH/$NADP^+$ determine the intracellular redox potential, affecting the thermodynamic driving force of many reactions, in particular providing electrons for lipids and deoxyribonucleotide and reducing oxidized precursors to maintain a reduced intracellular condition and ameliorate oxidative damage (Figure 2). The IDH1 and IDH2-dependent reaction is reversible, while IDH3-dependent reaction is

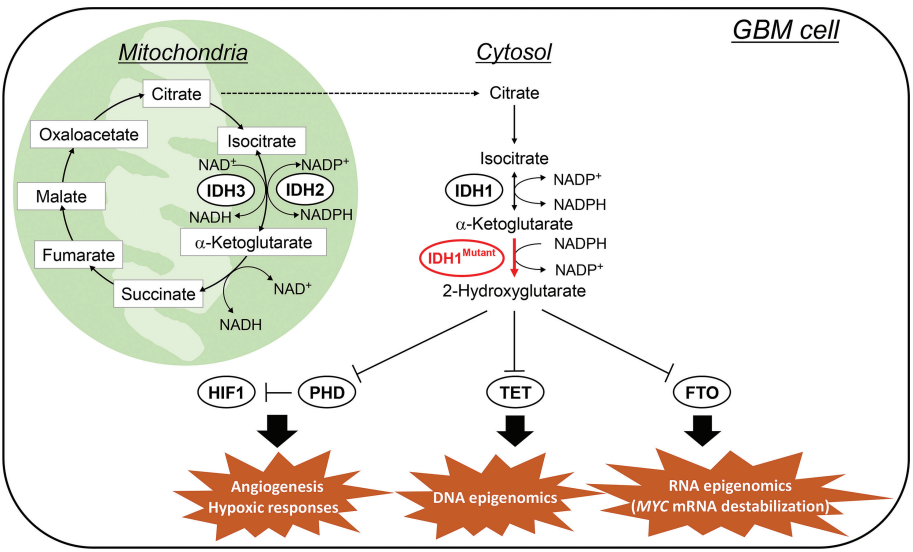


Figure 2. D-2HG generated by *IDH1* mutation interferes various pathways resulting in glioblastoma exacerbation. Citrate, the mitochondrial metabolite, flows out to cytosol. Isocitrate, synthesized by cytosolic citrate, is used as a substrate for the IDH1-mediated catabolism. The mutant IDH1 produces D-2-hydroxyglutarate (D-2HG) as an oncometabolite. In glioblastoma, accumulated D-2HG causes (i) angiogenesis and hypoxic responses through depression of HIF1 by PHD inhibition; (ii) reprograming of DNA epigenomics through TET inhibition; and (iii) reprograming of RNA epigenomics including destabilization of *MYC* mRNA through FTO inhibition. D-2HG, 2-hydroxyglutarate; FTO, fat mass and obesity-associated protein; HIF1, hypoxia inducible factor 1; IDH1, isocitrate dehydrogenase 1; PHD, prolyl hydroxylase domain -containing protein (PHD); TET, ten-eleven translocation enzyme.

irreversible (12–14). The reversible nature of IDH1 and IDH2 reaction plays an important role in reductive carboxylation, which enables cells lipogenesis under the conditions that decrease the TCA cycle coupled-oxidative phosphorylation (for example, hypoxia, VHL mutation) (15, 16) (Figures 1–3).

Wild-type IDH1 is overexpressed in many primary glioblastoma

Although wild-type *IDH1* has had much less attention compared to the research on glioma with *IDH1* mutation, several studies have revealed that wild-type IDH1 is overexpressed in several types of cancers, including non-small cell lung carcinoma (NSCLC) (17, 18), pancreatic adenocarcinoma (PDAC) (19), and primary glioblastoma (20, 21). Importantly, these studies show that IDH1 is overexpressed in over 60% of primary glioblastoma patients and is correlated with poor overall survival. Wahl et al. and Calvert et al. independently demonstrated that knocking down endogenous *IDH1* by shRNA, or pharmacological inactivation of IDH1 by the IDH1 inhibitor GSK864, decreases glioblastoma growth *in vitro* and extends survival of mice harboring intracranial glioblastoma, while overexpression of wild-type IDH1 shortened the survival of the glioblastoma mouse (20, 21).

Targeting the wild-type IDH to increase the therapeutic efficacy of radiation and chemotherapies

With regard to the primary glioblastoma, even with aggressive multimodal radiation and chemotherapy after surgery, only marginal improvements on survival are made (average of 2 months), with a median survival of just 14.6 months (22, 23). The use of tumor treating fields (TTFs) with the standard of care therapy in glioblastoma (IR plus TMZ) in a randomized open-label trial of 695 glioblastoma patients, reporting that median progression-free survival was 6.7 months in the TTF plus standard of care group versus 4 months in the standard of care group alone (24). Recent studies suggest that the IDH1 enzyme is a potential clinical target for glioblastoma therapy (25). The rationale is that IDH1 activity is considered to increase cytoplasmic NADPH/$NADP^+$ ratios, which promotes lipid biosynthesis and increases cellular defense against oxidative stress. Suppression of IDH1 activity could alter cellular metabolism, potentially lowering the ratio of NADPH/$NADP^+$, which sensitizes cells to oxidative stresses (Figure 3). Given that radiotherapy induces cell death through induction of reactive oxygen species (ROS) and DNA lesions (26, 27), targeting the IDH1 enzyme in glioma with wild-type *IDH1* allele (for example, primary glioblastoma) has exciting therapeutic potential.

Wahl *et al.* further demonstrated that knocking down wild-type *IDH1* in primary glioblastoma cell lines (wild-type IDH1) decreases the ratio of NADPH/$NADP^+$, as well as levels of deoxynucleotides and reduced glutathione (GSH) and increases the efficacy of radiation. This radiosensitization effect of IDH1 knockdown is reversed by treatment of anti-oxidant N-acetyl cysteine and/or nucleotide precursors, pointing that IDH1-dependent NADPH production is critical for glioblastoma radioresistance (20, 28). Likewise, suppression of mitochondrial IDH2 also increases radiosensitivity of primary glioblastoma cells (29). Furthermore, IDH1 inhibition decreases GSH and NADPH levels in the glioblastoma initiating

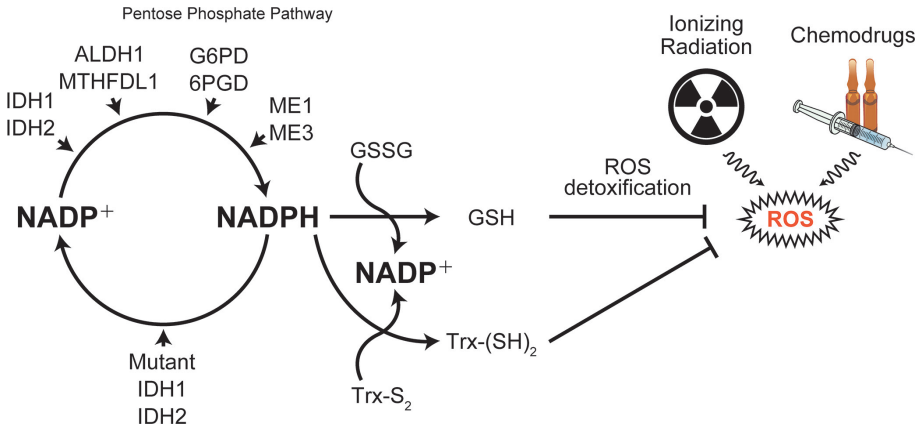


Figure 3. Effects of wild-type and mutant IDH enzymes on redox status and therapeutic efficacy. IDHs mutation may increase therapeutic efficacy of radiation and chemotherapies. IDH1, 2, and the other enzymes reduce $NADP^+$ to NADPH. NADPH also reduces GSSG or Trx-S_2 to GSH or Trx-$(SH)_2$, respectively. GSH and Trx-$(SH)_2$ detoxify ROS and decrease the effect of the radiation or chemotherapy treatment for glioblastoma patients. The decrease of NADPH/$NADP^+$ ratio by the inhibition of IDH enzymes has potential to increase the efficacy of the current treatment for glioblastoma. 6PGD, 6-Phosphogluconate dehydrogenase; ALDH1, aldehyde dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; GSSG, glutathione disulfide; IDH, isocitrate dehydrogenase; ME, malic enzyme; $NADP^+$ /NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; Trx, thioredoxin.

cells/glioblastoma stem cells carrying EGFR-amplification, making them more susceptible to EGFR inhibitor treatment (30). Serum-free culturing techniques have revealed a sub-population of "glioblastoma initiating cells" that may have increased radiation resistance and lead to recurrence after radiation treatment (31–34). The possibility of better targeting these cells via IDH1 inhibition may lead to better radiation response and delayed recurrence (22–24).

BIOLOGICAL IMPACT OF D-2HG ON GLIOBLASTOMA

A point mutation in the *IDH1* gene was initially identified through exome sequencing of colon tumor and glioblastoma samples (2, 35). Mutations in *IDH1* mostly occur at Arg-132 residue (R132) located within the catalytic domain, which is the binding site for isocitrate. R132H is the most common alteration, comprising >80% of all *IDH1* mutations in gliomas. Surprisingly, a study undertaking metabolomics analysis shows that the mutation of IDH enzymes bestows a new enzymatic function of reducing alpha-ketoglutarate (αKG) to D-2-hydroxyglutarate (D-2HG, or R-2HG) using NADPH as an electron donor (5). In the presence of the *IDH1* mutation, the D-2HG molecule, which is normally found at minute levels, can increase to millimolar amounts (5). D-2HG generated by *IDH1* mutation interferes various pathways resulting in glioblastoma exacerbation (Figure 2). Understandably, there has been considerable interest in what role this potential new "oncometabolite" might have on cells. Following the discovery of the *IDH1* mutation, many investigators sought to determine what new malignant traits this

mutation would bestow upon a cell. Unexpectedly, it appeared that in general, the addition of the *IDH1* mutation led to slower growth in most brain tumor models (36). This was a perplexing result and was hypothesized to be the reason why *IDH1* mutant tumors had a better prognosis compared to glioblastomas without the *IDH1* mutation. Extensive studies have shown the complexity of the IDH1 mutational effects and important issues affecting the interpretation of past research. The impact of D-2HG on transcriptional landscape, with particular emphasis on the recent discovery of the new effect of D-2HG on RNA epigenomics, is discussed below.

D-2HG induces epigenetic alternations by increased DNA and histone methylation

Even though mechanistic understanding of *IDH* mutations and D-2HG effects on gliomagenesis remain to be clarified, compelling evidence from Turcan *et al*. shows that the glioma-associated *IDH* mutation promotes hypermethylation of histone and DNA through its accumulated product D-2HG (37) (Figure 2). Mechanistically, because of its structural similarity to αKG, it has been considered that supra-physiologically elevated D-2HG levels inhibit enzymes, such as DNA demethylase ten-eleven translocation enzymes (TETs) and histone lysine demethylases (KDMs) that utilize αKG as a co-substrate (Figure 2). Consequently, *IDH1* mutation provokes epigenetic reprogramming of the transcriptional landscape of glioma (8, 38–41).

D-2HG inhibits RNA demethylase, FTO, leading to aberrant RNA methylation and growth suppression

While the dynamic covalent modifications (for example, methylation) to DNA and histones play critical roles in regulating gene transcriptions, an emerging research area is epigenetic regulation of RNA. Over 160 different chemical modifications in RNA have been identified (42). Among them, N^6-methyladenosine (m^6A) has been considered the most prevalent modification of RNA Pol II transcripts (43–45). In general, m^6A modification is enriched near the 5' untranslated terminal region (UTR) as well as the stop codon and 3′ UTR, which regulates mRNA transcription (46), splicing (47), export (48), stability (49), and translation (50, 51). Like methylation on DNA and histones, m^6A is a reversible modification, and fat mass and obesity-associated protein (FTO) is the first RNA demethylase identified for the removal of the methyl group of m^6A in mRNA with some extent to m^1A in specific tRNAs using αKG as a co-substrate (52, 53).

Accumulating evidence shows that m^6A mRNA modification is critical for glioblastoma stem cells self-renewal and tumorigenesis. Though there is some apparent discrepancy in terms of the role of methyltransferase responsible for m^6A in glioblastoma (48–50), the consensus is that inhibition of FTO significantly suppresses glioblastoma stem cells on culture and intracranial growth of glioblastoma stem cells in a mouse xenograft model (54–56). Importantly, our recent study has uncovered that D-2HG is a potent inhibitor for the FTO activity *in vitro* and *in vivo*, leading to the aberrant accumulation of m^6A mRNA in leukemia and glioma cells expressing *IDH1* mutant (57). In leukemic cells, FTO inhibition by D-2HG

decreases stability and thus, expression levels of *MYC*, one of the master regulators of hyper-anabolism and cell proliferation (57) (Figure 2), though whether this mechanism can be extended to glioma remains to be clarified. The results of our study revealed a surprising functional link between FTO and *IDH* mutations that could potentially explain why *IDH* mutated tumors bear proliferative disadvantage.

POTENTIAL IMPACTS OF *IDH1* MUTATION ON CELLULAR METABOLISM

The fact that *IDH1* mutant tumors carry a better prognosis than those without *IDH1* mutations has led to the hypothesis that this new enzyme may have deleterious effects on cellular metabolism. In accordance with this hypothesis, our study has shown that intracellular metabolism in *IDH*-mutated glioblastoma is significantly different from that in *IDH* wild-type glioblastoma, in particular, prominent in nucleotide metabolism pathway (58). To investigate this hypothesis, many investigators have overexpressed the *IDH1* mutant gene and then performed mass spectroscopy to examine the differences between parental and transformed lines. However, the results so far are mixed and need further studies to interpret the results. Here, we briefly summarize the experimental results, which appear controversial in some cases, and discuss the technical challenge of faithfully recapitulating the *IDH1* mutated glioma cellular status.

All studies to date have found that the addition of the *IDH1* mutant enzyme comes with a metabolic burden that makes the cell less fit and resilient. The first studies focused on the fact that the *IDH1* mutant enzyme was converting large amounts of αKG into D-2HG, making the assumption that αKG was derived largely from glutamine. Taking these facts together, it was hypothesized that the *IDH1* mutation led to cells becoming glutamine deficient. Seltzer et al. confirmed this by showing that the addition of the *IDH1* mutant enzyme made cells more vulnerable to glutaminase inhibition (59). Another set of studies focused on the fact that the *IDH1* mutant enzyme consumes one molecule of NADPH and produces a molecule of $NADP^+$ and, therefore, might have an effect on the level of ROS. Results on this topic have been mixed and sometimes appear controversial. Attempts to knock-in the *IDH1* mutation under a Nestin neural stem cell driver were embryonic lethal, but the salvaged cells had lower levels of ROS (60). In contrast, overexpressing the *IDH1* mutant enzyme in U87MG cells increased ROS levels and made the cells more vulnerable to radiation (61).

More recently, it was discovered that *IDH1* has an important role in reductive carboxylation, which is the ability of the cell to convert glutamine to citrate without going through the TCA cycle. This allows the cell to participate in lipogenesis and membrane synthesis in a hypoxic environment. The presumption would be that with a mutation in the *IDH1* enzyme, the native function of the enzyme would be diminished. Again, results have been mixed, with Grassian et al. reporting that overexpression of the *IDH1* mutant gene inhibited the ability of cells to perform reductive carboxylation under hypoxia (62). On the other hand, Reitman et al. using the same cell line found that the *IDH1* mutation actually facilitated the

ability of the cell to convert glutamine into fatty acids (palmitate) under hypoxic conditions (63).

A potential caveat and technical challenge to investigate the roles of IDH1 mutation on cellular metabolism

All of these studies suffer from two methodological problems. The first is that the metabolic effect of the *IDH1* mutant enzyme depends on the baseline metabolic background of the host cell. There is increasing evidence that the *IDH1* mutation is likely one of the first mutations to occur in gliomagenesis (64) and thus over many rounds of cell growth and selection, the cells have time to adjust and adapt to whatever metabolic effects the *IDH1* mutation may have. If the *IDH1* mutation were as detrimental to cellular function as studies suggest, the *IDH1* mutation would be deleted along with the other genes that impede accelerated cellular growth, for example, *PTEN, p53,* and *NF1*. The second is that these studies fail to answer the metabolic effect of blocking the *IDH1* mutant enzyme in a glioma cell that already has it.

In order to address these shortcomings, our group performed genetic and metabolic profiling on a panel of patient-derived *IDH1* wild-type and *IDH1* mutant glioblastoma cultures and compared these results with *IDH1* mutant overexpression models to determine the accuracy and differences of these models (58). We found that *IDH1* wild-type glioblastoma cells had a high genetic expression of *de novo* nucleotide synthesis genes and disproportionately shunted glucose through the pentose phosphate pathway for *de novo* nucleotide synthesis. In contrast, *IDH1* mutant glioblastoma cells were enriched for DNA repair response genes. Consistent with these predictions, *IDH1* wild-type glioblastomas were more vulnerable to *de novo* nucleotide synthesis inhibitors, and *IDH1* mutant glioblastomas were better able to repair DNA after radiation (58), which is also supported by our other studies (65–67). Initially, we assumed that the observed changes in transcriptome and metabolism were due to the direct metabolic effect of *IDH* mutation. However, surprisingly and importantly, there was no difference seen in nucleotide synthesis when the *IDH1* mutant enzyme was overexpressed on an *IDH1* wild-type background or when D-2HG production was blocked by an *IDH1* mutant inhibitor (58).

Similar to previous studies, overexpression of the *IDH1* mutant enzyme depleted TCA cycle intermediates and led to much slower growth (58). In contrast, inhibiting D-2HG formation in endogenous *IDH1* mutant cells had no effect on either growth or the level of TCA cycle intermediates. Furthermore, the baseline levels of TCA cycle intermediates were roughly equal between the *IDH1* mutant and *IDH1* wild-type cultures (58). Taken together, these results suggest that the *IDH1* mutation has different effects on different cellular backgrounds and is largely well tolerated in endogenous *IDH1* mutant glioma cells. Although *IDH1* mutant and *IDH1* wild-type gliomas have different metabolic vulnerabilities, these may be largely due to their differences in growth speed and genetic contexts after the long-term gliomagenesis. Further dedicated studies are needed to clarify whether the *IDH1* mutation by itself is sufficient to induce the metabolic complexity and heterogeneity of *IDH* mutated glioma.

THREE POSSIBLE MODELS EXPLAIN THE APPARENTLY PARADOXICAL OUTCOMES OF *IDH1* MUTATION

Why would a mutation that slows growth be selected over neighboring cells without that mutation and a presumably faster rate of growth? We raise three working models.

Model 1. Increased stress resilience by IDH1 mutation

One possibility is that the *IDH1* mutation enables cells to resist death or anti-growth signals in their microenvironment. This theory is supported by the discovery that the D-2HG molecule could inhibit the function of alpha-ketoglutarate-dependent enzymes by outcompeting alpha-ketoglutarate (23). his led to the hypothesis that the *IDH1* mutation might give a cell the ability to resist environmental influences and prevent differentiation from a progenitor cell to a more differentiated and less prolific cell type. In several cellular contexts, notably fat cells (8), chondrocytes (68) and liver cells (61), overexpressing the *IDH1* mutation in precursor/stem cells prevented those cells from differentiating. In each of these previous studies, there was a key mediator gene that was essential for differentiation. During differentiation, this gene was activated by the demethylation of a key histone mark in the promoter or enhancer region. In the presence of the *IDH1* mutation or high levels of 2-HG, this histone demethylation was prevented, and the cell failed to differentiate and instead maintained its proliferative potential. However, trying to show that the *IDH1* mutation has the ability to block differentiation in neural cells has been more elusive. Overexpressing the *IDH1* mutation in a mouse sub-ventricular zone (SVZ) stem cell culture changed the default differentiation from a GFAP-positive astrocyte to a TUJ1 positive neuron; however, it did not prevent differentiation or lead to increased growth (8).

Model 2. Chronic malignant evolution via epigenomic repression

A second possibility is that the effect of the *IDH1* mutation is slow but over time can gradually convert the epigenetic state of a cell to a more malignant phenotype. The most popular model of this theory is that the *IDH1* mutant enzyme impairs the ability of the TET enzymes to demethylate DNA. In this case, the activity of the DNA methyltransferase (DNMT) enzyme family is unopposed and leads to a gradual increase of methylation throughout the genome. The methylation of CpG islands, particularly in key regulatory regions, tends to decrease the expression of those genes. Over time more and more tumor suppressor genes would become repressed until the cell becomes tumorigenic. The strongest evidence for this theory comes from a study using overexpression of the *IDH1* mutant enzyme in an astrocyte line. This led to the gradual induction of Nestin expression and a small increase in growth over many passages. This long-term *IDH1* mutant expression was also associated with increased DNA and histone methylation (37).

Model 3. IDH1 mutation acts as a mutator

A third possibility is that the *IDH1* mutation predisposes to further mutations. When studying patients with *IDH1* mutant tumors, it was noted that following resection, when the tumor eventually grew back, the tumors had often acquired a new set of mutations. Mutations that were present in the first tumor were not present in the second tumor. The only mutations that were always present were *IDH1* and p53 (64). This result implies that the *IDH1* mutation is likely the initial mutation in gliomagenesis and is sufficient to generate enough mutations for tumorigenesis multiple times throughout a patient's life. However, first, p53 must be rendered non-functional. It is not clear how the *IDH1* mutation leads to further mutations. One possibility is the methylation and down-regulation of DNA repair machinery, in particular the gene encoding O-6-Methylguanine-DNA Methyltransferase (*MGMT),* a DNA repair enzyme removing the guanine-alkyl group induced by alkylating agents such as temozolomide. *IDH1* mutation and *MGMT* methylation are correlated, although there are *IDH1* mutant tumors with unmethylated MGMT (69, 70). As additional evidence, while MGMT methylation is an independent predictor of a positive response to temozolomide in *IDH1* wild-type glioma cells, it is not a predictor of chemotherapy response in *IDH1* mutant cells, implying that either MGMT itself or the MGMT pathway may be non-functional in *IDH1* mutant cells (71). Another possibility is that the *IDH1* mutation may lead to higher levels of endogenous ROS, predisposing to DNA damage. While presumably the *IDH1* mutant enzyme would lead to an alteration in the NADPH/$NADP^+$ equilibrium, it is not obvious *a priori* what effect this would have on total endogenous ROS levels. Consequently, the question of whether the addition of the *IDH1* mutation to cells causes an increase or a decrease in ROS levels is still a matter of debate, with different studies showing conflicting results (60, 61).

THERAPEUTIC SENSITIVITY AND RESISTANCE OF *IDH* MUTANT GLIOMAS

Standard therapy for a newly diagnosed glioblastoma involves maximal safe surgical resection, temozolomide, and fractionated radiation. This protocol has been validated by randomized controlled trials (22, 23). However, these trials were based on a mixed cohort of *IDH1* mutant and *IDH1* wild-type patients. Presumably, given demographics and prevalence, the majority of these patients were *IDH1* wild-type. This means that the results of these trials may not necessarily translate to *IDH1* mutant gliomas. *IDH1* mutant gliomas are associated with longer survival, and some have assumed that this is due to a better response to adjuvant therapy (temozolomide and radiation) (72). However, arguing against this assumption is the observation that *IDH1* wild-type gliomas show increasing therapeutic response and increased survival to higher doses of temozolomide, whereas *IDH1* mutant gliomas do not show any improvement with higher doses of temozolomide (73). Due to this concern, additional chemotherapy regimens were tried, and recent trials have shown that CCNU is effective in combination with radiation in *IDH1* mutant low-grade gliomas (74). These clinical trials present

multiple logistical difficulties related to the low incidence of the disease and the relatively long and variable survival.

As many such questions remain unanswered, the most relevant and perhaps most controversial is the question of whether *IDH1* mutant gliomas are more or less sensitive to radiation than their *IDH1* wild-type counterparts. Multiple studies have found conflicting results that seem to depend on the cell line model used and even the culture conditions. Studies using serum culturing conditions found that the *IDH1* mutation is associated with increased radiation sensitivity in both overexpression (61, 75) and endogenous (76) *in vitro* studies. In contrast, overexpression (75) and endogenous models (58) grown in serum-free conditions show radiation resistance. There is a shortage of mouse models of *IDH1* mutant gliomas; however, in one of the few studies to utilize a mouse model, the *IDH1* mutation was associated with radiation resistance via upregulation of DNA damage response genes (77). The general clinical consensus is that radiation is effective against *IDH1* mutant gliomas, and no randomized clinical trial is to test this assertion is forthcoming.

Small molecule *IDH1* mutant inhibitor

Following the discovery of the *IDH1* mutation, there was a great deal of interest in developing mutant IDH targeted therapy, leading to a series of potent small molecule inhibitors against mutant IDH1 (for example, AGI-5198) and IDH2 enzyme (e.g., AGI-6780) (Figure 4) (78). In the case of the leukemia model TF-1, mutant IDH2 specific inhibitor AGI-6780 prevented the changes seen following IDH2 mutant expression and induced differentiation of the *IDH2* mutated leukemic cells (79). Consistent with these findings, early clinical trials with similar inhibitors in acute myeloid leukemia are also promising (80). After taking the *IDH1* mutant inhibitor, patients with *IDH1* mutant acute myeloid leukemia show a progressive decrease in the number of immature tumor-type myeloid cells with a corresponding increase in mature differentiated cells. Most encouragingly, unlike traditional chemotherapy, there is no myelosuppression seen across the other myeloid lineages.

However, in the case of *IDH1* mutant glioma models, the results were more mixed. The first attempt to treat an *IDH1* mutant glioma with the inhibitor was met with some success. Treatment of mutant IDH1 inhibitor AGI-5198, the first prototype inhibitor (78, 81), decreased glioma size and increased expression of GFAP, suggesting differentiation (82). However, later attempts to repeat this data have failed. In one of the more thorough studies, Tateishi et al. treated *IDH1* mutant cells with AGI-5198 for over a year and found no difference in either DNA methylation or histone modification, and there was a slight increase in growth with the addition of AGI-5198 (83). These variable results from the preclinical studies are, in part, likely due to the poor metabolic stability and low blood-brain barrier penetrance of the compound (78).

One difficulty with mutant IDH1 inhibitor to treat brain tumor models is the issue of time. While Turcan et al. was able to demonstrate an increase in methylation with the addition of the *IDH1* mutant enzyme, the effect required the cells to undergo 40 passages and presumably several hundred cell divisions (37). Even then, the majority of the newly methylated sites were only partially methylated.

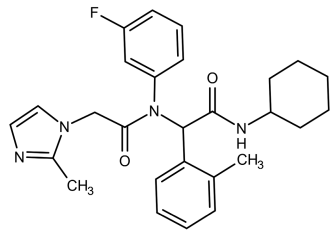
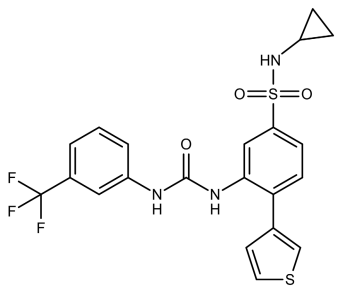
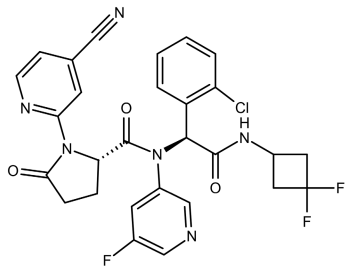
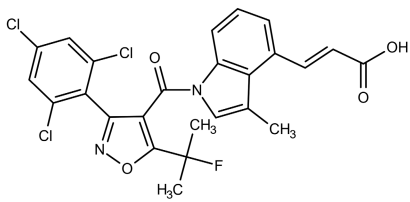
	Target	Generation	Chemical structure
AGI-5198	IDH1-R132H/C	1st	
AGI-6780	IDH2-R140Q	1st	
AG-120	IDH1-R132H/C	2nd	
DS-1001b	IDH1-R132H/C	2nd	

Figure 4. Potential molecules for treatment of IDH mutated cancers. The chemical structure of inhibitors of IDH mutants are shown. AGI-5198, AG-120, and DS-1001b target the IDH1 mutants (R132H and R132C), and AGI-6780 targets an IDH2 mutant (R140Q). AG-120 and DS-1001b are expected as the next-generation therapeutics for curing glioblastoma.

Any study that fails to find an effect of adding the *IDH1* mutant enzyme can be criticized for not giving the cells enough time no matter how much time was given. One possibility for the disagreement between the two studies is that while the *IDH1* mutation is able to induce methylation, once the methylation is induced, it is irreversible. It is also difficult to translate these results into a clinical context where the vast majority of cells in the brain are post-mitotic.

Nevertheless, given the potential for targeted efficacy with limited off-target toxicity, many IDH1 mutant inhibitors have entered clinical trials. AG-120/Ivosidenib is showing a good safety profile and a trend for tumor stability in non-enhancing tumors (84) (Clinical Trials: NCT02073994; NCT03343197). Furthermore, clinical trials with another next-generation compound with greater blood-brain barrier penetration, DS-1001b (85), are currently enrolling for glioma patients with an *IDH1* mutation (NCT03030066; NCT04458272) (Figure 4).

CONCLUSION

In this chapter, we introduced the impact of wild-type and mutant *IDH1* on glioblastoma metabolism, growth, and current therapeutic approach. Since its discovery nearly a decade ago, the *IDH1* mutation has fast become one of the most well-known and complicated metabolic mutations found in cancer. Convincing evidence exists that it is the initial mutation that begins the process of tumorigenesis. Despite the difficulty of modeling its behavior *in vitro*, significant strides have been made to link the derangement in metabolic function to its deregulation of epigenetics and, finally, its effect on growth. Overexpression models of *IDH1* mutant function likely over-estimate the negative effects of the mutant enzyme on growth and metabolic function. In several studies, inhibiting the *IDH1* mutant enzyme in endogenous cultures seems to have minimal effects on either growth or the metabolic state of the cell. Our study also demonstrates the tumor-suppressive effect of the accumulated D-2HG by FTO inhibition. However, all studies demonstrate *IDH1* mutant and *IDH1* wild-type gliomas have different metabolic properties, pointing that they may have distinctive vulnerabilities allowing for the possibility of personalized therapy. Collectively, these results suggest that further and broader investigation of the mechanistic role of these enzymes in *IDH1*-wild-type and mutant glioma is warranted.

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5

Integration of Molecular Analysis, Cutting-edge Mouse Genetic Models and Proton Therapy to Improve Outcomes for Glioma Patients

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Abstract: Despite recent advances in general cancer treatment, glioblastoma remains among the most lethal of human malignancies. Even with aggressive multimodal radiation and chemotherapy after surgery, glioblastoma recurs with a bleak prognosis. Decades of research focused on strategies such as increasing radiation sensitivity and interference with oncogenic signal transduction have yielded only incremental improvements at best. This is due in part to the radioresistance of glioblastoma and molecular heterogeneity of tumor cells. We hypothesize is that the development of more effective glioblastoma therapies will require: (i) a more accurate molecular analysis of glioblastoma so as to predict response to therapy; (ii) better genetically engineered mouse models, which can faithfully recapitulate human glioblastoma and the tumor microenvironment to test new approaches and (iii) development and application of more accurate and focused methods to deliver sustained high energy particles to glioblastoma tumor sites. This chapter describes the current state-of-the-art molecular analysis approaches, latest in glioma mouse modelling, and advances in the application of proton therapy treatment and research. By integrating basic and clinical research with cutting-edge technologies, a mechanistic understanding of glioblastoma therapy resistance and pathogenesis and the development of new therapeutics to overcome the therapeutic resistance of glioblastoma will be advanced.

Keywords: brain tumor-associated edema; *in utero* electroporation-based glioma mouse models; mycophenolic acid; proton beam therapy; single-cell RNA sequence

INTRODUCTION

Glioblastoma is a WHO Grade IV primary brain cancer with an abysmal prognosis (1). The current gold standard initial treatment approach is a gross resection of the tumor guided by the use of 5-Aminolevulinic Acid (5-ALA), a porphyrin precursor, to identify the infiltrative margins (2). Such emerging imaging approaches combined with advances in surgical techniques have improved surgical outcomes, but the undetected residual microscopic disease remains a significant problem. Gross surgical resection, when anatomically possible, is followed by a one month break and then chemoradiation and ionizing radiation (IR) induces DNA double-strand breaks through direct high-energy damage to the sugar backbone of DNA, but also through free radicals generated in cells, which accounts for 60–70% of DNA lesions, exerting its genotoxic and ultimately cytotoxic effect (3, 4). However, glioblastoma is intrinsically resistant to IR (5–13), and therefore ionizing radiation therapy yields only marginal improvements in patient survival (14, 15), with a nearly 80% rate of recurrence within the high dose radiation field (16, 17). The nature of the resistance is unclear, but the general consensus is that it is related at least in part to increased glioblastoma tolerance to reactive oxygen species (11, 12, 18–20) and enhanced GTP metabolism (21–23).

Stupp and colleagues described a therapeutic approach that remains the standard of care therapy for glioblastoma. The “Stupp protocol,” as it is commonly

called, uses IR plus concomitant and adjuvant temozolomide (TMZ, Temodar) (15). The addition of temozolomide, DNA-damage based treatment through alkylating guanine and adenine bases of DNA, to radiation, improves median survival by approximately an additional 6 months (15). It was noted that *MGMT* methylated glioblastomas fared better in terms of survival than *MGMT* unmethylated glioblastomas. These studies indicate that the efficacy of IR can be significantly improved by combination therapy. These results also point to the importance of mechanistic understanding of radioresistance and the identification of a new pathway that more effectively increases the efficacy of radiation on the tumor but not normal tissue.

Recently, Stupp and colleagues described the use of tumor treating fields (TTFs) with the standard of care therapy in glioblastoma (IR plus TMZ) in a randomized open-label trial of 695 glioblastoma patients, reporting that median progression-free survival was 6.7 months in the TTF plus standard of care group versus 4 months in the standard of care group alone (21). Phase 3 clinical trials of bevacizumab (Avastin) in glioblastoma showed no survival advantage; however, in glioblastoma patients with steroid dependence, bevacizumab can be used as a steroid-sparing agent (22). After the significant investment of resources into the upfront clinical trial setting of glioblastoma with immunotherapies, neoadjuvant anti-PD-1 therapy provided a promising survival benefit in recurrent glioblastoma (23), however, the Phase III study was negative in terms of survival benefit. Many vaccine trials are on the horizon for glioblastomas, and these are promising for patients who fulfill the selection criteria for these studies. However, many glioblastoma patients do not qualify for vaccine studies due to the location of the tumor, the immunogenicity of their tumor, and their performance status (24, 25). A critical challenge has been to develop new ways for accurate and rapid prediction of an individual patient's susceptibility to treatment.

Identifying a number of molecular genetic (for example, *p53*, *NF1, PTEN, PDGFR*) and signaling pathways (for example, RAS/ERK, PI3K/AKT pathways) involved in cancer development has led to several targeted agents being investigated in clinical evaluation for glioblastoma (26–28). To test the effects of the identified pathway and drug, preclinical mouse models have proven to be an invaluable tool, but unfortunately, none of these targets have been translatable in the clinical arena. Glioblastoma mouse models allow one to investigate basic mechanisms—enabling precise examination of numerous aspects, including cellular origins, regional differences in microenvironments, and the function of specific genetic events. However, a major challenge has been to develop preclinical mouse models that recapitulate the human glioblastoma nature in a time and cost-effective fashion. Furthermore, gliomas encompass a diverse set of tumors that can differ in location, age of onset, mutation status, and histopathological features making this an extraordinary challenge.

So, how can we change the prognosis of a patient with a glioblastoma? What target(s) should we be investigating as possible therapeutic vulnerabilities? How can we overcome the various barriers to generate and use glioblastoma mouse models? This chapter will review new advances in radiation therapeutics, molecular analysis of glioblastomas, and new animal models that will address these questions.

THE CURRENT STATE OF MOLECULAR ANALYSIS AND RELEVANCE IN GLIOBLASTOMA TREATMENT OPTIONS

When a glioblastoma tumor is resected surgically, the tumor cells can be an invaluable source of information that can (and should) be utilized to guide patient treatment (personalized medicine). The tumor cells could potentially be used in a number of platforms, including RNA expression analysis (29–36), protein characterization (37–41), and metabolic profiling (37–39) (Figure 1). Once targets are identified, appropriate models can be generated to test treatment strategies, including 3D platforms such as *ex vivo* Organ-on-Chip (40, 41), *in vitro* cell culture methods including spheroids and organoids, and of course mouse models, transgenic and/or intracranial xenograft (37, 42–44) as well as large animals (for example, canine) (45, 46). Below we detail a newer emerging mouse model approach. Here we detail analysis of RNA sequencing to identify therapeutic targets and RNA-based therapeutics to target the mRNA or non-coding RNA.

Analysis of mRNA in glioblastoma

The genomic analyses of the tumor tissue or whole cells significantly increased our understanding of GBM in patients. For example, several whole-genome DNA sequencing studies reported (47) resulted in subtyping of glioblastomas based on their molecular phenotype. The *IDH1/2*-mutant GBMs are distinct categories, for example, mutations in the *EGFR, PDGFRA, NF1* genes; mutations in *hTERT* promoter; epigenetic changes such as altered methylation of the *MGMT* gene promoter (48, 49). While advanced genetic analysis has improved the prediction of glioma outcomes and treatment approaches (50–52), unpredictable significant inter-individual variation in therapeutic efficacy is often seen in the treatment of glioblastoma patients. Apart from the tumor itself, glioblastomas are heterogeneous, composed of diverse cells, such as immune cells and stromal cells (53, 54).

Two of the significant challenges are understanding the pathogenesis of glioblastoma and predicting more accurately patient sensitivity to a selected treatment(s). The analysis of individual parts of the genome and the transcribed RNA provides the potential for a more comprehensive understanding of the pathogenesis of glioma and possible treatments tailored to individual patients. The recent development of single-cell RNA-sequencing (scRNA-seq) has enabled functional analysis of individual glioblastoma cells. The differential transcriptional landscape between individual cells can have profound functional consequences, for example, cellular, molecular, genetic, epigenetic, and metabolic heterogeneity. These heterogeneities affect the course of glioblastoma development and therapeutic responses, challenging personalized medicine. Historically, single-cell transcriptional analysis is started by single-cell quantitative PCR (qPCR). Rapid progress in the development of sequencing and single-cell isolation technologies in recent years has enabled RNA sequencing at single-cell levels (55). The scRNA-seq technology can uncover highly complex as well as rare cell populations. Also, the time-resolved scRNA-seq analysis can clarify the regulatory relationship between gene expression in responses to stimuli (for example, therapeutic treatment) and track the trajectories of cell lineages.

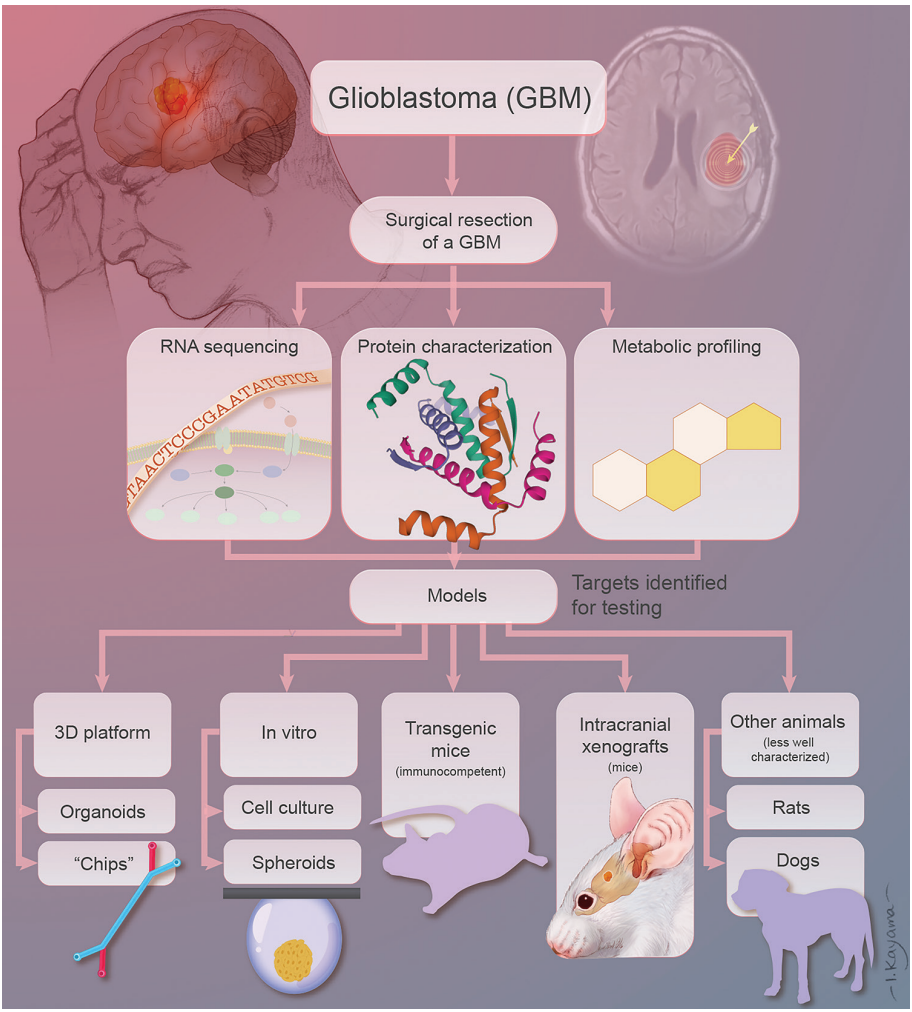

Figure 1. A flow diagram of a personalized medicine approach of a patient's glioblastoma tumor. This diagram shows how a patient with a glioblastoma experiencing headaches, has imaging that localizes a tumor. When the tumor is resected surgically, it could potentially be used in a number of platforms, including, RNA sequencing, protein characterization and metabolic profiling. Once targets are identified the appropriate models can be generated as shown in the flow diagram, including, 3D platforms, *in vitro* cell culture methods, transgenic mice, intracranial xenografts and large animal studies.

Suva and colleagues (56) utilized scRNA-seq analysis of 28 glioblastomas and bulk expression analysis of 401 specimens from the TCGA, combined with functional approaches and single-cell lineage tracing to create a cohesive model for the genetic heterogeneity and cellular states in glioblastomas. They found that malignant cell niches in glioblastomas are influenced by copy number amplifications of *CDK4*, *EGFR*, and *PDGFR* loci and mutations in *NF1*. Couturier *et al.* (57), utilizing scRNA-seq analysis of 53,586 adult glioblastoma cells and 22,637 normal

human fetal brain cells, reported a conserved neural tri-lineage hierarchy centered around glial progenitor-like cells. They reported that within this progenitor population, most cancer cells are centered around said glial progenitor-like cells and that the course of glioblastoma development is along with the conserved neurodevelopmental gene programs, which possesses a rapidly dividing progenitor population. Their scRNA-seq analysis revealed new insight on primary glioblastomas and created a hierarchical map to identify therapeutic targets specific to progenitor cancer stem cells.

Analysis of non-coding RNA in glioblastoma

While there is an emphasis placed on the study of the expression of protein-coding genes (mRNA transcripts), it is essential not to overlook the potential contribution of non-coding genes as well as promoter and enhancer elements. Functional studies in disparate systemic cancers have shown that long non-coding RNAs and miRNAs can promote pathogenesis. Integration analyses of long non-coding RNAs and competing endogenous RNA networks in glioblastomas are much rarer. These networks may be the key to understanding the pathogenesis of glioblastomas, as well as predicting the therapeutic efficacy of the selected treatment, thus representing an untapped therapeutic potential (58).

RNA-based therapies and their delivery to a glioblastoma

We suspect that many potential targets will not have targeted drugs available. Thus, we should keep open the possibility of targeting transcripts and/or non-coding RNAs with RNA-based therapeutics (such as siRNAs, ADARs, gRNA for a CRISPR-Cas9 approach) and the development of technologies that can aid their delivery to a glioblastoma. On this front, an emerging clinical technology that may significantly impact glioblastoma treatment is the application of focused ultrasound combined with microbubbles to facilitate the delivery of RNA-based therapeutics as well as large drugs and monoclonal antibodies into glioblastomas (59, 60). Importantly, this non-invasive technology can overcome the blood-brain/blood-tumor barriers for delivery of RNA-based therapeutics and the nanoparticles used to aid their delivery, but many agents that might be effective *in vitro* but because of their size/mass cannot cross the blood-brain/blood-tumor barriers, for example, earlier generations of EGFR tyrosine kinase inhibitors and newer agents such as monoclonal antibodies and vaccines (61, 62).

EMERGING MOUSE MODELS OF PEDIATRIC AND ADULT HIGH-GRADE GLIOMA AND GLIOBLASTOMA

Developing animal models that faithfully recapitulate the features of human gliomas is essential for conducting accurate preclinical studies that facilitate the development of novel drugs and therapeutic strategies that can be translated into the clinic. While the establishment of patient-derived cell lines and xenograft models has dramatically advanced our understanding of this deadly disease, limitations

for patient-derived models include the inability to establish consistent cultures from tumors, the presence of numerous and complex genetic events, and the use of immunodeficient hosts, which limits their use for immunotherapy studies (63). Traditionally, immunocompetent glioblastoma mouse models have been generated using the knock-out and transgenic mouse approach. However, the knock-out and transgenic-mouse approach often require crossing with other mutant mice (for example, *Trp53*-KO, *Pten*-KO), which takes a number of years and costs to maintain the colonies (63). The replication-competent ALV splice acceptor (RCAS)/*tv-a* glioblastoma system developed by Holland's group overcomes this issue (64, 65). The engineered RCAS virus carrying the gene of interest (for example, EGFR, shRNA for PTEN, sgRNA for p53) can be delivered to the specific cells (for example, glia) that is engineered to express its receptor *t-va* (64, 65). While the RCAS/*t-va* system is a revolutionized technology, potential limitations are that the RCAS virus vector typically allows the small size of the insert (3 kb), and it requires *t-va* expressing transgenic mouse; thus, the system is basically incompatible with the existing mutant mouse. In this section, we highlight *in utero* electroporation (IUE) based glioma mouse models, which have been developed for both adult and pediatric gliomas (Figure 2).

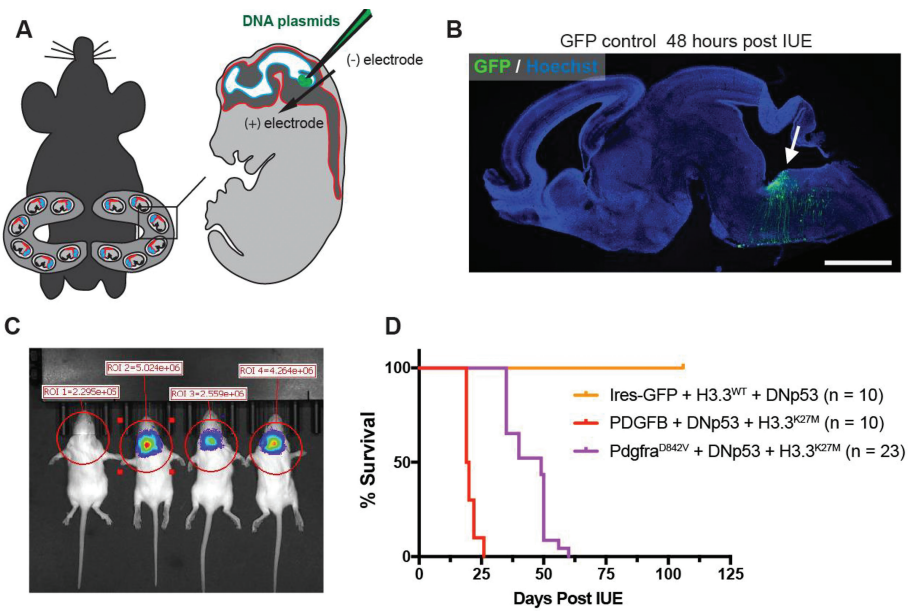

Figure 2. ***In utero*** **electroporation-based models of DIPG. A.** Schematic cartoon of the IUE procedure targeting the developing embryonic brainstem. **B.** Representative image of GFP-positive transfected brainstem neural stem and progenitor cells 48 hours post brainstem IUE in a sagittal brain section. **C.** Example of luciferase positive and negative IUE pups at two weeks postnatal. **D.** Survival curve for different oncogenic IUE combinations depicting different rates of tumorigenesis.

In *utero* electroporation-based glioma mouse models

To address some of the drawbacks of traditional genetic mouse models, including high cost and lengthy development times, a number of groups have utilized IUE as a flexible and rapid platform to develop glioma mouse models. IUE of the developing brain was first described by Tabata and Nakajima in 2001 (66) and has traditionally been used to label and track cellular migration or the impact of transient genetic manipulations on cellular proliferation and differentiation (67). More recently, a number of laboratories have paired this technique with advances in DNA editing technologies to develop new brain tumor mouse models (68–70). First, the application of CRISPR-Cas9 technology to directly edit the mouse genome has been successfully used to generate adult glioma mouse models (68). By transfecting guide RNAs (gRNA) targeting known glioma mutations, such as *Nf1*, *Trp53*, and *Pten*, along with the Cas9 protein, Zuckermann *et al*. first described this efficient method to create triple loss-of-function mutant adult glioma mouse models using IUE (68). They validated the effectiveness of CRISPR-Cas9 gRNAs at generating frameshift INDELs within targeted genes; successfully targeted IUE offspring developed fully penetrant gliomas. This triple-CRISPR model has been used by a number of other groups, providing a platform to screen genetic variants (71, 72) and study functional interactions between tumor cells and the microenvironment (73).

The ability to restrict transfection to specific brain areas with IUE also provides an opportunity to model regionally distinct glioma subtypes, such as diffuse intrinsic pontine glioma (DIPG), a highly lethal pediatric glioma that arises in the brainstem (74–76). Compared to adult gliomas, DIPGs harbor unique genetic alterations, including histone K27M mutations that are found in approximately 80% of cases (77). The use of traditional genetic mouse models to model histone mutations did not result in glioma formation, yet the introduction of histone K27M mutations, paired with *Tp53* loss-of-function and *Pdgfra* expression by cortical or brainstem targeted IUE produced fully penetrant gliomas (78). Additional IUE based DIPG mouse models generated by Patel *et al.* revealed a range of latencies, histopathologies, and gene expression changes induced by specific combinations of mutations used to model DIPG (70). In this study, they noted the importance of histone K27M mutations in accelerating glioma formation in the presence of $Pdgfra^{D842V}$ + DN-p53 (dominant-negative p53) and its requirement for tumor formation in WT-Pdgfra + DN-p53 backgrounds. Besides hastening glioma development, H3 K27M mutations drove epigenetic and transcriptional changes that mirror those identified in K27M mutant DIPG patient samples, including loss of H3K27me3 (79) and decreased CDKN2A (p16) expression, respectively (80, 81).

Developing animal models that recapitulate the features of human gliomas is essential for conducting accurate preclinical studies to improve the prediction of drug penetration and efficacy, as well as radiation efficacy. Utilizing the IUE platform to generate regionally and genetically distinct glioma mouse model in a rapid and flexible manner provides exciting new research possibilities. This includes investigating recently identified mutations discovered by next-generation sequencing studies of patient samples or targeting DNA transfection to specific cell-types to test the impact of cellular origin on tumorigenesis. In addition, the immune competency status of these IUE glioma mouse models also provides an *in vivo*

system to examine immune-modulatory and immunotherapy strategies. Our recent studies have shown that an IUE-based high-grade glioma is a powerful approach, revealing that the efficacy of dasatinib treatment of $Pdgfra^{D842V}$ + DN-p53 high-grade glioma is enhanced with everolimus (82). The results suggest a promising route for improving targeted therapy for high-grade glioma patient with the driver mutation of PDGFα and p53.

PROTON BEAM THERAPY

Theoretically, higher doses of radiation increase the anti-glioma effect. However, an increase in dose is generally associated with an increased risk of radiation necrosis and toxicity to normal surrounding tissue (83). Various technological advances have led to improved delivery systems in radiotherapy. Contact radiotherapy via radioactive sources and superficial energies was superseded by 2-dimensional radiotherapy with the ability to generate increasing energies of electrons and generated photons. This advanced with the advent of computed tomography to 3-dimensional treatment. Intensity-modulated radiotherapy, improving conformality, and advanced planning techniques were made possible by computer technology improvements (84). In addition, significant advances in the treatment of malignancies were made in the ability to use various particles. In this section, we focus on proton beam therapy (PBT), one of the most precise and advanced forms of radiation therapy available in the world today.

Stereotactic radiosurgery

Stereotactic radiosurgery may also be considered in the upfront or the more commonly recurrent setting for glioma treatment. This is an ablative therapy of very high radiotherapy doses administered over traditionally one, but up to five fractions. In the upfront setting, consideration is primarily focused on decreasing the number of fractions received, typically spanning six weeks for 30 total treatments (85). For treatment at the time of relapse, both fractionated and stereotactic radiotherapy may be considered. As at the time of presentation, fractionation allows for the treatment of larger volumes commonly associated with these infiltrative tumors. Yet, radiosurgery may be appealing for both conveniences as well as it is biologically appealing to overcome some of the radioresistant mechanism of gliomas (86). Future directions within radiotherapy in glioma treatment will continue to improve upon advanced planning and delivery systems. In addition, combinatorial therapies maximizing efficacy and minimizing toxicity continue to be investigated.

Proton beam therapy leads to fewer side effects and complications

Following photon radiation (ionizing radiation), proton radiation has become the predominant modality used in the treatment of brain tumors. Due to the relatively low mass and lack of charge, photons slowly lose energy along a path length extending through the entirety of the patient. Conversely, due to high relative mass and associated charge, protons have a dose deposition concentrated near the

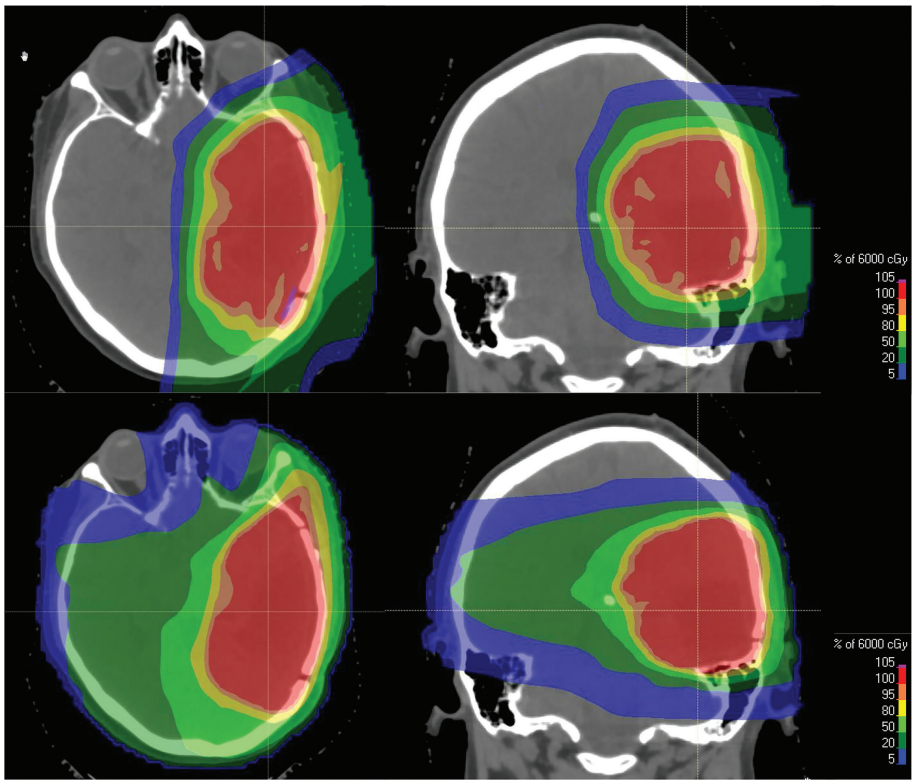

Figure 3. Comparative dosimetry for left temporal glioma radiotherapy treatment with protons (upper) and photons (lower). Prescription is for 60 Cobalt Gray Equivalent in 30 fractions. Noted decrease in radiotherapy exposure to the eyes, optic apparatus, brainstem, and contralateral structures.

end of their range, allowing the bulk of radiation dose deposited in a highly confined area, termed the "Bragg peak" (Figure 3). The Bragg peak is the characteristic dose deposition profile of a proton with nearly all energy loss just prior to its end of the range (87). This allows for a decrease in radiation exposure downstream from the target tissue, decreasing the side effects and complications of radiotherapy.

The superiority in the conformality and accuracy of proton beam therapy is a critical advantage for the treatment of brain tumors, circumventing the side effects and potentially allowing for an increase in the administered dose to the tumor. Proton beam radiotherapy has the capability to reduce the dose to non-tumor tissue in virtually all tumor locations within the brain (88, 89). The magnitude and clinical benefit of these reductions are variable and require clinical determination by the radiation oncologist. In the pediatric setting where radiation exposure to developing tissue is of the highest concern, studies have shown detriment to cognition and neuroendocrine function from radiotherapy exposures (90). Dosimetric studies have confirmed a decrease in radiation exposure to

structures associated with memory and cognition utilizing proton radiotherapy (91). Early studies reporting neurocognitive outcomes in pediatric patients undergoing proton beam therapy correlate these dosimetric changes to favorable outcomes compared to similar photon historical cohorts (92, 93).

The use of proton radiotherapy in adult brain tumors was constrained by the availability of these machines and was, therefore, initially reserved predominantly for tumors considered radioresistant, thus requiring higher doses. Representative tumors include skull base chordoma and chondrosarcoma, and uveal melanoma, as well as some benign processes (94). With the expanding number of facilities increasing access to proton radiotherapy, indications for proton radiotherapy have similarly increased. Brain tumors of favorable prognosis in the adult population are now considered for proton radiotherapy to minimize long-term toxicity. In addition, proton radiotherapy may be considered in cases of reirradiation where critical structures may have been previously exposed to doses at the threshold of significant risk for toxicity or to minimize the quality of life detriment from large cumulative exposures (95).

Proton beam therapy center with a capability for basic research

Proton beam therapy has been used clinically for over 50 years. However, proton beam therapy was only available for a few populations of patients due to its cost and limited availability. Thanks to significant advances in technology, there are now 31 proton therapy centers operative in the USA today. As the numbers of proton centers increase, there has been more consideration for the basic research requirements that would expand the potential application of proton therapy and improve its efficacy. Towards this end, Cincinnati Children's Hospital invested $120 million and launched one of the most advanced proton research and cancer treatment centers in the world in 2016. The facility includes several tracks for basic biological research to investigate the cellular responses of tumors to proton radiation and for translational research to develop and refine other treatments that can augment proton therapy. While the majority of current research is undertaken by cooperation among the local entities (Cincinnati Children's Hospital Medical Center and the University of Cincinnati), collaborations across countries and continents are widely open in many directions and ongoing.

CONCLUSION

The current standard of care for glioblastoma is surgical resection followed by radiation combined with temozolomide. Adjuvant therapy is vital because glioblastoma grows invasively in the surrounding brain tissue and, almost invariably, is rarely completely resectable. Patient response to treatment is often unpredictable and can differ significantly from experimental results in mice. A critical challenge is to develop new ways for accurate and rapid prediction of an individual patient's susceptibility to treatment. In this chapter, we have discussed three approaches to achieve this goal: (i) new genomic and RNA molecular analysis, (ii) an enhanced glioblastoma mouse model using IUE technology, and (iii) improved radiotherapy using proton therapy.

Integrating sophisticated pathway analysis with mouse models that recapitulate the human disease coupled with the ability to perform basic studies on the mechanisms of photon therapy has a high potential to overcome the current challenges in GBM therapy. While this needs an establishment of a framework to proceed with a sample obtained from a patient and link the result of the approaches, once established, we expect that the combination of these three approached with the current standard of care will enable choosing the best treatment and markedly improve glioblastoma outcomes.

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6

Cholesterol Derivatives as Promising Anticancer Agents in Glioblastoma Metabolic Therapy

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Abstract: Malignant brain tumors are among the most devastating types of cancer. Glioblastoma is the most common and serious form of brain cancer. Most glioblastomas are surgically unresectable and are typically diagnosed at an advanced stage. The high level of resistance to chemotherapy, radiotherapy and immunotherapy makes glioblastoma one of the most difficult cancers to treat. In brain tumors, the challenges of targeted therapy also include the blood-brain barrier, which often contributes to treatment failure. Therefore, developments of new treatment strategies are required. Metabolic treatments could be an alternative to conventional therapies. Metabolic approaches aim at suppressing glioblastoma tumorigenicity leading to glioblastoma cell death. Since cholesterol metabolism is deregulated in these tumors, this is a promising potential target for therapy. As glioblastoma cells draw on cholesterol from the central nervous system to survive,

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their growth is theoretically unlimited. Targeting the metabolism of cholesterol by different strategies using, among others, targets of LXRs (Liver X Receptors) or toxic cholesterol analogues could potentially oppose the growth of glial tumors. This chapter discusses the potential of targeting cholesterol metabolism using cholesterol derivatives as a pharmacological alternative to current therapeutic strategy.

Keywords: cancer metabolic therapy; cholesterol derivatives; cholesterol metabolism; glioblastoma; oxysterols

INTRODUCTION

Most of central nervous system (CNS) cancers are found in the brain while others develop in the meninges, spinal cord, and cranial nerves (1). The origin and location of brain tumors determine their type. Primary brain cancers originate in the brain which is also a frequent site for secondary or metastatic tumors. Gliomas are the most common primary tumor of the CNS (2). According to the World Health Organization (WHO), gliomas are traditionally classified based on the cell type of origin: astrocytic, oligodendroglial, oligoastrocytic, or ependymal tumors (3, 4). The current classification system is a grading system that grades tumors from grade I (benign) to IV (highly malignant) based on increasing cellular density, nuclear atypias, mitosis, vascular proliferation and necrosis (5). Glioblastoma is the most aggressive diffuse glioma of astrocytic lineage and is considered a grade IV glioma (4), making up 54% of all gliomas and 16% of all primary brain tumors (5). Glioblastoma is characterized by an aberrant metabolism which has important roles in carcinogenesis, metastasis, drug resistance, and cancer stem cells. Cancer cells adapt their metabolism in response to signals from the microenvironment and proliferation (6). Therefore, overcoming metabolic alterations is an important goal of modern cancer therapeutics.

CANCER METABOLISM

Aberrant metabolism is a major feature of cancer that directly affects tumor signal transduction pathways and cellular reactions. The metabolic heterogeneity and plasticity of cancers results from genetic heterogeneity and cancer microenvironment. Oncogenic signal pathways including Hippo, PI3K-AKT/mTOR, Myc, p53 and LKB1-AMPK play an important role in the regulation of cancer metabolism (7). Hence, overcoming metabolic plasticity constitutes a therapeutic challenge. Cancer cells modify their metabolic pathways, maximizing the expression and the efficiency of metabolic enzymes activities to meet their increased needs and to overcome cancer microenvironment which induces chronic nutrient deficiency and oxygen concentrations reduction (8,9). Respiratory mechanisms in cancer cells are still under investigation. Warburg effect states that respiratory mechanisms are damaged especially in the mitochondria and that cancer cells obtain ATP through glycolysis instead of oxidative phosphorylation (10), while other

data argue that the cancer cells produce energy using oxidative phosphorylation and their mitochondria is intact (11, 12). As a result of glycolysis and oxidative phosphorylation, glutamine becomes the main source of NADH and FADH2 giving rise to upregulated glutaminolysis in cancer cells (13). Fatty acids do not merely have roles as structural components but are also vital for cell response and cancer cell proliferation. Fatty acid synthesis is upregulated in tumors (14). Cancer cells compensate for fatty acid synthesis by up-regulating external lipid absorption instead of using *de novo* fatty acid synthesis because fatty acid synthesis is an oxygen-consuming process (15, 16). This upregulation overcomes the metabolic barriers that restrict the synthesis of metabolites (7). Reactive oxygen species (ROS) have been spotted in practically all cancers, where they influence cancer microenvironment and also promote many aspects of cancer development. Their contribution to carcinogenesis is still debatable and is evidently highly complex (17). Therefore, understanding the cellular metabolism that oversees ROS-related signaling will offer appreciated visions to target cancer cells. Aberrant cancer metabolism including aerobic glycolysis, increased glutamine, and fatty acid anabolic metabolism, are not simply outcomes of aberrant signal pathways, but potentially contribute to cancer cell proliferation, metastasis and drug resistance (7). The metabolic therapy involves the bypass of cancer metabolism. It may affect sensitivity of the cancer cells to anticancer drugs and may allow them to avoid the non-specific cytotoxicity of these drugs and overcome drug resistance. This treatment approach avoids metabolic plasticity, which is the capacity of cells to adapt their metabolic status to their specific needs (18). Therefore, understanding cancer metabolism and identification of new drugs targeting it may yield new therapeutic opportunities. However, metabolic heterogeneity and plasticity make this approach difficult. One highly heterogeneous cancer for which current therapies utterly fail is the deadly brain cancer glioblastoma.

GLIOBLASTOMA FEATURES

Glioblastoma is the most common and lethal primary brain cancer that expose an implacable malignant progression characterized by expanded invasion throughout the brain, resistance to therapeutic strategies, devastation of normal brain tissue, and death (7).

Epidemiology of glioblastoma

According to the Global Burden of Disease Study in 2016, at the global level, there were 330,000 cases of CNS cancer, with an age-standardized incidence rate of 4.63 per 100,000 person-years and with an age-standardized death rate of 3.24 per 100,000 person-years (1). Glioblastoma, the most common primary brain cancer of glial origin, is almost universally fatal with a median age of 64 years (19). Incidence of CNS cancers peaks in early childhood (<5 years of age) and increases after 15 years of age, with no difference in incidence rates by sex during childhood, but a diverging incidence between sexes with increasing age, leading to 1.6 times higher incidence in men than women (20), though this difference was not considered significant (1).

Risk factors for glioblastoma

Few known risk factors are associated with CNS cancers; the only positive association being with ionizing radiation (for example, previous therapeutic irradiation) (21, 22). Various genetic syndromes and associated low frequency alleles are associated with increased risk of CNS cancer, but these account for only a minute fraction of total cases (23, 24). Glioblastoma has been associated with the viruses SV40 (25), HHV-6 (26, 27), and cytomegalovirus (28). Uncommon risk factors have been considered, including smoking and pesticide exposure (29).

Antigenic and genetic characteristics of glioblastoma

The characterization of molecular alterations in glioblastoma could contribute to optimal therapeutic strategies. Various prognostic markers have been identified in glioblastoma, including methylation status of the gene promoter for O^6-methylguanine-DNA methyltransferase (MGMT), isocitrate dehydrogenase enzyme 1/2 (IDH1/2) mutation, epidermal growth factor receptor (EGFR) overexpression and amplification, glioma-CpG island methylator phenotype (G-CIMP), tumor protein 53 (TP53) mutation and genetic losses of chromosomes (30). Two models of progression have been proposed based on the molecular alterations in glioblastoma: primary (or *de novo*) glioblastoma and secondary glioblastoma. Primary *de novo* glioblastomas come from astrocytes or precursor/stem cells that have baseline mutations (31). Primary glioblastomas are frequently found to overexpress EGFR, and less frequently show mouse double minute 2 (MDM2) amplification, high frequency of telomerase reverse transcriptase (hTERT) promoter and p16 deletions, loss of heterozygosity on10q, phosphatase and tensin homolog gene (PTEN) mutations while TP53 mutation is infrequent (5, 30, 31). Secondary glioblastoma develops from a pre-existing low-grade glioma. They are characterized by TP53 mutation and alpha thalassemia X-linked mental retardation syndrome (ATR-X) (3, 30). Moreover, in addition to these mutations, they may present with the same molecular alterations as *de novo* glioblastoma. Many other genetic alterations have been described in glioblastoma, and the majority are found in two pathways: the retinoblastoma protein (RB), and the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) (32). Glioblastoma has alterations in 68–78% and 88% of these pathways, respectively (33). Glioblastoma-O is a rare subtype of glioblastoma with an oligodendroglioma component. It has longer survival when compared to other glioblastomas (30, 34). According to the 2016 WHO classification, glioblastoma is classified based on the status of IDH mutation into three groups: glioblastoma IDH-wild type, which represents about 90% of glioblastomas (including giant cell glioblastoma, gliosarcoma, and epithelioid glioblastoma); glioblastoma IDH-mutant, which represents 10%; and glioblastoma NOS (glioblastoma IDH-Not Otherwise Specified), in cases where IDH status was not sought or is not possible to confirm) (4, 35, 36). The classification of gliomas (3, 4, 37) is summarized in Figure 1.

Glioblastoma pathogenesis

Glioblastoma is generally located in the supratentorial region and rapidly infiltrates the brain parenchyma, sometimes becoming very large before producing

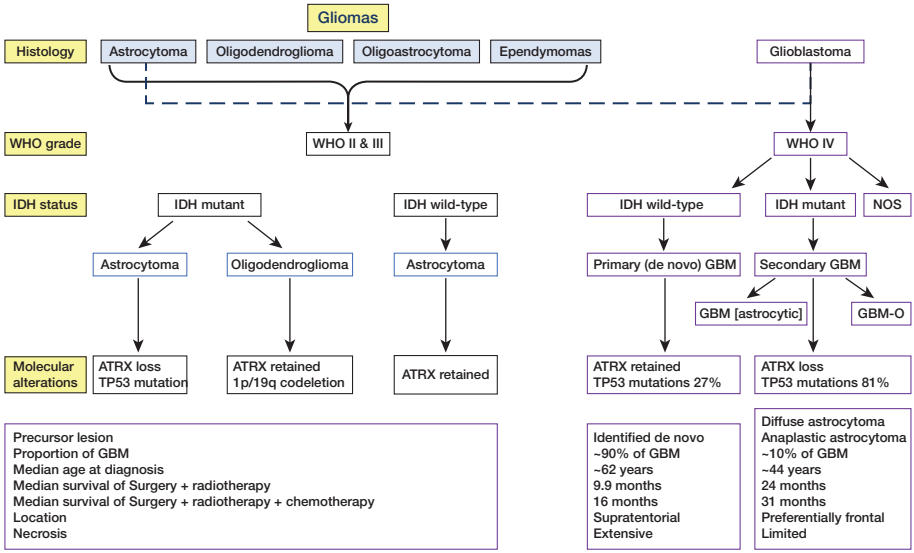

Figure 1. Classification of gliomas. Classification based on antigenic and genetic characteristics, and according to World Health Organization (3,4,37).

symptoms (31). Metastases of glioblastoma beyond the CNS are extremely rare (35). Glioblastoma is characterized by the presence of hyperplastic blood vessels that present with disrupted morphology and functionality (38), with small areas of necrotic tissue surrounded by anaplastic cells. The increased hypoxia within glioblastoma leads to cancer progression by promoting processes such as immunosuppression (38, 39). The invasive nature of glioblastoma may be explained by: (i) the upregulation of ion channels with gene alterations (40); (ii) the oncometabolite D2-hydroxyglutarate (D-2-HG) that accumulates in the tumor cell that modifies the tumor epigenome (hypermethylation of histones and DNA) and promotes tumor initiation and progression (41); and (iii) the behavior of IDH1-mutated glioblastoma cells that invade into healthy parts of the brain where glutamate concentrations excreted by healthy astrocytes are higher (42). The invasive nature of glioblastoma, with its cellular properties similar to progenitor cells, make complete removal of glioblastoma by surgery difficult, and this could be the possible cause of resistance to conventional treatments (43).

METABOLISM IN GLIOBLASTOMA

Abnormal metabolism is an emerging feature of glioblastoma with alterations to glycolysis, oxidative phosphorylation, the pentose phosphate pathway, amino acid metabolism as well as lipid oxidation and synthesis (6). Lipid metabolism pertinent to cancer is an actionable anticancer target. *De novo* lipid synthesis can feed proliferating tumor cells with phospholipid components (44, 45). Furthermore, the upregulation of mitochondrial β-oxidation can favor cancer cell

energetics and redox homeostasis (46). Lipid-derived messengers have also an important role in the regulation of major signaling pathways and the coordination of immunosuppressive mechanisms (47, 48). Thus, lipid metabolism involves a variety of oncogenic processes including carcinogenesis, metastases, and drug resistance (49–51).

Cholesterol metabolism in glioblastoma

Understanding the role of cholesterol metabolism and transport in glioblastoma cells and the underlying mechanisms of cholesterol-related drug resistance could lead to the development of more effective, targeted therapies for glioblastoma. The cholesterol pathway has emerged as a potential target for glioblastoma amenable to targeted pharmacologic treatment (52). Brain cholesterol represents 20–25% of total body cholesterol (53). However, peripheral and CNS cholesterol metabolism are regulated independently. The dynamics of the brain cholesterol pool and its metabolism is distinct from other organs due to the inability of peripheral cholesterol to cross the blood-brain barrier (54). Peripheral cholesterol depends on the balance between dietary intake and hepatic synthesis and degradation, whereas in the CNS, cholesterol is synthetized *de novo* by astrocytes and delivered to neurons as well as to glioblastoma cells (55, 56). Cholesterol provided by the astrocytes is a crucial step for growth and survival for glioblastoma cells (54). The cholesterol produced and secreted by astrocytes is supplied to the glioblastoma cells by apolipoprotein E (Apo-E). Oxysterols and other cholesterol derivatives produced in neurons following cholesterol uptake and metabolism can be physiological agonists for liver X receptors α/β (LXR) (52). Oxysterols inhibit cholesterol synthesis and enhance its export by activating LXRs (57, 58). Activation of LXR results in its dimerization with retinoid X receptor (RXR), favoring cholesterol efflux through sterol transporters such as ATP-binding cassette A1 (ABCA1) which is the main exporter of cholesterol bound to Apo-E, and the suppression of cholesterol uptake through MYLIP also known as IDOL (inducible degrader of the LDL receptor) (54, 59, 60). The E3 ligase IDOL is transcriptionally up-regulated by LXR/RXR in response to an increase in intracellular cholesterol (61). IDOL targets the low-density lipoprotein receptor (LDLR) for degradation (60). The LXR-IDOL-LDLR mechanism results in a decrease in cholesterol uptake, thereby regulating the level of intracellular cholesterol (54) (Figure 2). In glioblastoma cells, these cholesterol regulatory and surveillance mechanisms occurring in normal glial and nervous cells are disrupted (52, 54).

CURRENT GLIOBLASTOMA THERAPIES

Patients with CNS cancer often present with a spectrum of non-specific symptoms. There is no screening test available for CNS cancer that allows early and consistent detection (62). Because of the invasive nature of glioblastoma, the entire tumor cannot be removed surgically (63). Optimal treatment combines biopsy or aggressive surgical resection with postoperative radiation and chemotherapy (64). Despite optimal treatment, glioblastoma usually recurs. Only countries with advanced health care systems can provide highly specialized radiotherapy

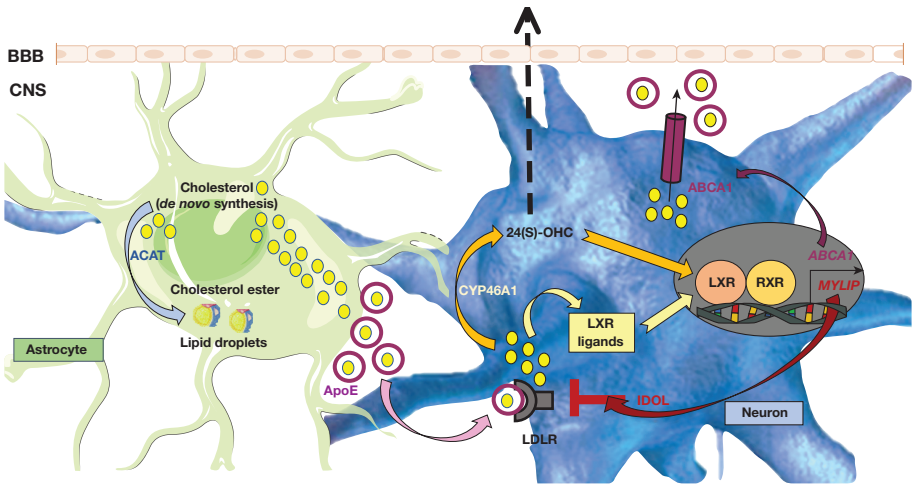

Figure 2. Regulation of cholesterol metabolism in brain neurons. Peripheral and CNS cholesterol metabolism are regulated independently. In the brain, the cholesterol produced *de novo* and secreted by the astrocytes is provided by Apo-E to neurons. Endogenous LXR ligands are oxysterols and other cholesterol derivatives produced in neurons following cholesterol uptake and metabolism. The main sterol transporter ABCA1 and the E3 ligase IDOL are transcriptionally up-regulated by LXR/RXR in response to an increase in intracellular cholesterol, resulting in inhibition of the expression of LDLR and in a decrease in cholesterol uptake, thereby lowering the level of intracellular cholesterol. In GBM cells, these mechanisms are disturbed. The GBM cells are unable to produce sufficient endogenous LXR ligands, especially oxysterols, thus promoting exogenous cholesterol uptake and intracellular accumulation of cholesterol which contributes to cell proliferation (52,54). BBB, blood brain barrier; CNS, central nervous system.

and neuro-oncology services (65). Glioblastoma is one of the hardest to treat cancer due to its high level of resistance to conventional therapies, without forgetting the contribution of the blood-brain barrier to treatment failure (66).

Glioblastoma is diagnosed at an advanced stage and has a low survival rate of 12 to 15 months on average, with fewer than 3–7% of people surviving longer than five years (67) and without treatment, survival is typically around three months (68). Radiation and temozolomide (TMZ) chemotherapy are used after surgery to destroy what was unable to be removed surgically, and recurring tumors. TMZ is an alkylating agent; TMZ is a triazene derivative, which undergoes rapid chemical conversion at physiological pH to the active monomethyl triazenoimidazole carboxamide (MTIC). Glioblastomas are well known to contain areas of tissue with hypoxia, which are highly resistant to radiation. New research approaches are looking into the use of an oxygen diffusion-enhancing compound, trans sodium crocetinate (TSC), as radiosensitizer (69). Currently, chemoradiotherapy gives the best overall survival, but is associated with a greater risk of adverse events than radiotherapy alone (70). TMZ seems to work by sensitizing tumor cells to radiation, and appears more effective for tumors with MGMT promoter methylation (71). Glioblastoma therapeutic failure including immunotherapy has been attributed, among others, to its intrinsic heterogeneity and to the immune microenvironment which is considered as a major obstacle to generating

an effective antitumor immune response (72,73). Therefore, developments of new treatments are required. Metabolic treatment could be an alternative to conventional therapies.

THERAPIES TARGETING CHOLESTEROL METABOLISM

Cancer arises by mutations within oncogenes and tumor suppressor genes. These genetic mutations regulate the expression and activity of several proteins involved in the control of cell growth including metabolic enzymes which are considered attractive drug targets (7). Antimetabolites which are small molecules that inhibit the activity of enzymes involved in nucleotide base synthesis, are among metabolism-targeting drugs that have had clinical success (74). Though, nucleotide metabolism is only one of many metabolic dependencies altered to favor carcinogenesis (74). Because cholesterol metabolism involves in glioblastoma cells growth, the cholesterol pathway has emerged as a potential target for glioblastoma therapy. There are several approaches involving cholesterol metabolism known in the glioblastoma field, all of which have the same goal: the depletion of intracellular cholesterol leading to cell death.

Liver X Receptors (LXR)-E3 ligase IDOL-Low-density lipoprotein receptor (LDLR)

The LXR-IDOL-LDLR axis is a targetable pathway in glioblastoma (75). The LXR non-steroidal agonists GW3965 and LXR-623 up-regulate the expression of E3 ubiquitin ligase IDOL, which results in reduced LDLR levels. They also up-regulate the expression of the cholesterol transporter gene ABCA1, which then induces substantial apoptosis via activation of the LXRβ isoform (54, 75). With archazolid B, the expression of LDLR is upregulated, leading to an increase in extracellular cholesterol uptake. This drug hampers the action of V-ATPase due to a proton transport defect. This leads to associated increases in lysosomal pH, thereby preventing cholesterol recycling (76). The build-up of cholesterol within intracellular organelles makes it effectively unavailable for use by glioblastoma cells.

RNA-binding proteins (RBPs)

RNA-binding proteins (RBPs) have important roles in human biology. It has been reported that metabolic enzymes were identified as RBPs and participate in varied metabolic pathways including lipid metabolism (77). RBPs of glioblastoma are therefore another potential target. The expression and function of RNA binding proteins Fragile X-Related (FXR1) could be of interest in glioblastoma therapy. Downregulation of FXR1 or MIR17HG, also known as miR-17-92 which is the host gene for the miR-17-92a-1 gene cluster at 13q31 (78), results in inhibition of glioblastoma cells progression. The smallest tumor volumes and the longest survivals of nude mice *in vivo* were obtained with FXR1 knockdown combined with inhibition of MIR17HG (79).

Statins

It is also suggested that statins could be effective in preventing drug resistance in glioblastoma. The role of intracellular cholesterol flux in TMZ-induced cell death is still under investigation. Data are contradictory, some showing that statins reduced TMZ-induced cell death and therefore proposed the use of TMZ with soluble cholesterol which could potentially serve as combination therapy to treat glioblastoma (80), while other data proved that simvastatin promotes TMZ-induced apoptosis in glioblastoma cells (52). Statins may potentially serve as a new therapeutic approach for combination therapy in glioblastoma (81). The effect of statins may be due to autophagy modulated by the mevalonate pathway (82, 83), through geranylgeranylation of the small GTPase molecule Rab11 (82). Geranylgeranyl-pyrophosphate, which is produced by the mevalonate cascade, plays an important role in the prenylation of the superfamily of Ras-like GTPase proteins known as the Rab family (84). Rab GTPases are involved in vesicular trafficking, where Rab11 and Rab7 are critical components for autophagosome formation and autophagosome–lysosome fusion (85). Thus, autophagy flux is inhibited due to the decreased prenylation of Rab11 and Rab7, which is a result of the inhibition of mevalonate pathway by statins (84, 85). Therefore the inhibition of mevalonate pathway followed by autophagy inhibition leads to apoptotic cell death (83, 86). Long-term consumption of statins increased survival rate of various cancer patients (87). The same result was shown with glioblastoma patients (88). Cancers with overactive Myc, which is a transcription factor that regulates cholesterol synthesis, have been observed with amplified expression of HMGCR and sensitivity to statins (89, 90). Thus, inhibiting autophagy with statins or other molecules via the mevalonate pathway or other channels could also be a new approach to treat glioblastoma.

Sterol regulatory element-binding protein (SREBP)

Sterol regulatory element-binding protein (SREBP) may also be a novel therapeutic target. Intracellular levels of cholesterol and fatty acids are controlled through a feedback regulatory system mediated by SREBPs (91). SREBP-1a can activate all target genes. SREBP-1c primarily regulates fatty acid metabolism, such as by regulating the fatty acid synthase (FASN) gene. SREBP-2 is mainly responsible for cholesterol-related genes, such as the HMG-CoA reductase (HMGCR) and low-density lipoprotein receptor (LDLR) gene (92). Cholesterol and fatty acid synthesis decreases following the inhibition of SREBPs expression. Therefore, SREBP and its pathways can be novel targets for the treatment of glioblastoma (93). The oncogenic signaling EGFR-PI3K-Akt pathway is involved in boosting lipid levels and their uptake into glioblastoma cells by the upregulation of the sterol regulatory element-binding protein (SREBP-1) (94). Thus, inhibition of EGFR-PI3K-Akt signaling by the EGFR inhibitor lapatinib suppresses SREBP-1 nuclear translocation sensitized glioblastoma xenografts in mice, resulting in cell death (95). Phytol and retinol, inhibitors of SREBP-1 synthesis, are able to induce glioblastoma cell death by interfering with fatty acid and cholesterol metabolism (94). Betulin specifically inhibits the maturation of SREBP by inducing the interaction of SREBP cleavage-activating protein (SCAP) and insulin-induced gene (Insig), which leads

to the endoplasmic reticulum-retention of SCAP–SREBP complex. Betulin decreases the biosynthesis of cholesterol and fatty acids (92) and could lead to glioblastoma cell death. The flavanol quercetin decreased the expression of SREBP-1 and SREBP-2, decreasing the viability of glioblastoma cells (96). Oxysterols such as 22 (R)-hydroxycholesterol and 24 (S), 25-epoxycholesterol appear to inhibit cholesterol biosynthesis, possibly via their accumulation, which inhibits the cleavage of SREBP-2 (97).

Cholesterol derivatives: oxysterols

Cholesterol and its metabolites (precursors and derivatives) play an important role in cancer (98). Certain cholesterol metabolites such as estrogens and androgens can promote cancer, while others such as glucocorticoids suppress cancer (99). Oxysterols such as 7-ketocholesterol (7-KC) and 25-hydroxycholesterol (25-OHC) are products of cholesterol oxidation obtained mainly either by cholesterol auto-oxidation or enzymatic oxidation of cholesterol, respectively, and are potent suppressors of HMGCR activity (100, 101). Suppression of reductase prevents cells from synthesizing cholesterol which could inhibit cell growth (101). The chronological study of the cytotoxic activities of oxysterols has led to an interest in their activities on metabolism. Oxysterols and pro-drugs derived from oxysterols were initially studied for their cytotoxicity; mainly their ability to induce cell death. Then, due to their pro-inflammatory properties, their immunomodulatory-anticancerous properties were also examined. As some oxysterols can inhibit the activity of HMGCR, their ability to act on cholesterol metabolism was investigated. Oxysterols quickly emerged as interesting molecules in cancer due to their greatly altered levels in some tumors and due to their ability to promote cellular oxidative stress and cytotoxicity (102, 103). Currently, oxysterols and their involvement in cholesterol metabolism constitute a new field of research, and their implication in oncogenic pathways is also of interest, as some of them appear to have mutagenic properties (104).

Oxysterols can act on G protein-coupled receptors (GPCR) (e.g. Epstein-Barr virus-induced gene 2 [EBI2]), smoothened (SMO), chemokine (C-X-C motif) receptor 2 [CXCR2]), nuclear receptors (LXR, retinoic acid receptor-related orphan receptor (ROR), estrogen receptor [ERα]), anti-estrogen binding site (AEBS) (105) and through transporters or regulatory proteins (106). The mechanisms by which oxysterols may influence proliferation are manifold: two types of effects related to AEBS are the inhibition of cholesterol epoxide hydrolase (ChEH) (107, 108) and the inhibition of cholesterol biosynthesis (109), leading to increases in levels of cholesterol intermediates (110). Resulting sterol accumulation is associated with the development of autophagic features (111–114), and can lead to survival or lethal autophagy depending on concentrations and time of treatment (115). B-ring oxysterols, such as 7-KC, 7-ketocholestanol, and 6-ketocholestanol (116) bind to AEBS. Certain oxysterols can suppress the activation of SREBPs by binding to an oxysterol sensing protein in the endoplasmic reticulum, Insig (101–105). Some oxysterols can accelerate the degradation of the key cholesterol biosynthetic enzyme, HMGCR, and/or serve as natural ligand activators of LXR (103, 105, 117–119). Oxysterols have been shown to induce apoptosis in a variety of cell lines: human monocyte blood cells (U937), murine lymphoma cells (RDM4), human vascular endothelial cells (HUVECs), human artery smooth

muscle cells (A7R5), human colon cancer cells (Caco-2), chinese hamster ovary cells (CHO), mastocytoma cells (P815) and T cell derived human leukemia lines (CEM-C1 and CEM-C7) as well as on numerous types of nerve cells (158N, BV-2 and N2a) (104, 120–128). There are two major apoptotic pathways; the death receptor or extrinsic pathway (129, 130) and the mitochondrial or intrinsic pathway (131, 132). 27-hydroxycholesterol (27-OHC) has recently been shown to act as an estrogen receptor agonist in breast cancer, contributing to tumor growth and metastasis (133). To date, several works have concentrated on oxysterols oxidized at C7, in particular, 7-KC and 7β-hydroxycholesterol (7β-OHC). 7β-OHC derivatives, some blocked at C-3-OH group and others phosphodiesters of 7β-OHC, were synthesized and showed similar toxicity to their parent compound under *in vitro* conditions (127, 134). 7-KC and 7β-OHC are potent inducers of cell death and trigger apoptosis through the mitochondrial pathway on several cell types (135–139). 7-KC and 7β-OHC induce a mode of cell death defined as oxiapoptophagy (OXIdative stress + APOPTOsis + autoPHAGY) (140). Consequently, cholesterol derivatives and notably oxysterols, constitute an interesting class of molecules which are of huge interest in oncology, and may form a new class of antitumor agents.

Natural and synthetic cholesterol derivatives

We have exploited the anti-proliferative and immunosuppressive properties of cholesterol derivatives to study their effect on C6 cells which are the most common experimental models used in neuro-oncology to study glioblastoma (141–145). We have compared the cytotoxic effects of the following natural and synthetic cholesterol derivatives: natural compounds (7β-OHC, 22 (R)-hydroxycholesterol (22R-OHC), 24 (S)-hydroxycholesterol (24 (S)-OHC)). Synthetic compounds (22(R)-hydroxy-Δ^9-cholestanol (22R-ISO-OHC), 23-(4-Methylfuran-2,5-dione)-3α-hydroxy-24-nor-5β-cholane (LITHO 1a), 23-(4-Methylfuran-2,5-dione)-3α,7α-dihydroxy-24-nor-5(3-cholane) (CHENO 1b), 23-(4-Methyl-1H-pyrrole-2,5-dione)-3α-hydroxy-24-nor-5β-cholane (LITOMAL 7a), 23-(4-Methyl-1H-pyrrole-2,5-dione)-3α,7α, 12α-trihydroxy-24-nor-5β-cholane (COLMAL 7f) and ethanol maleimide derivatives of litocholic and chenodeoxycholic acid (LITOMET, CHENOMET)) (146,147). The sytematic name of LITOMET is (23-((2-hydroxyethyl)-4-methyl-1H-pyrrole-2,5-dione)-3α-hydroxy-24-nor-5β-cholane) and the systematic name of CHENOMET is (23-((2-hydroxyethyl)-4-methyl-1H-pyrrole-2,5-dione)- 3α,7α-dihydroxy-24-nor-5β-cholane). We evaluated the effects on cell morphology by phase contrast microscopy, on cell viability by the MTT test, on esterase activity by the FDA test, on cell survival by the clonogenicity test, on mitochondria by measuring the mitochondrial transmembrane potential (ΔΨm) by staining with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), on the plasma membrane also indicating cell mortality by propidium iodide (PI) staining, on lysosomes by acridine orange (AO) staining, on the cell cycle by detection of cells in phase (G2+M) after PI staining, on autophagy by quantification of LC3-II and LC3-I protein expression by Western blot (LC-3II/LC-3I ratio). PI, $DiOC_6(3)$ and AO staining were measured by flow cytometry. Based on these tests a multidimensional and multivariate heatmap was made (Figure 3). The heatmap obtained allows for a comparative study of the cytotoxicity of the cholesterol derivatives studied, some of which trigger a non-apoptotic mode of cell death with characteristics of autophagy leading an increase of the ratio LC3-IILC3-I. Our results underline that cholesterol derivatives, including oxysterols, are cytotoxic on tumor cells and can potentially constitute a new group of molecules to treat glioblastoma.

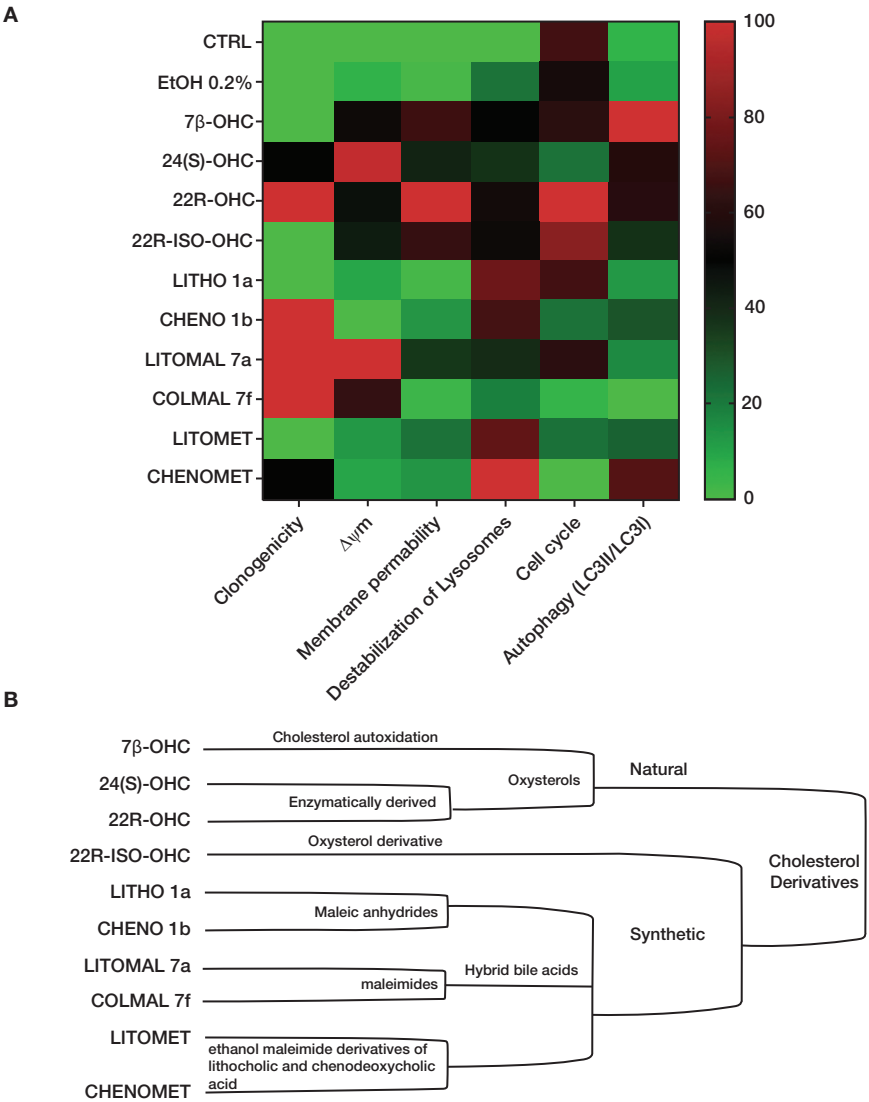

Figure 3. Heatmap and Cholesterol derivatives classification. A. The heatmap is a color-grading system comparing the effects of cholesterol derivatives on rat C6 glioblastoma cells. It grades from green (little or no effect) to red (maximum effect) based on clonogenicity, mitochondrial membrane potential (ΔΨm), permeability of the plasma membrane, destabilization of lysosomes, effects on the cell cycle and activity on autophagy measured by the LC3II/LC3I ratio. **B.** Classification comparing natural and synthetic cholesterol derivatives.

Mitochondria as therapeutic targets

Mitochondria and their increased cholesterol levels have been implicated in many pathological processes, including cancer (148, 149). Mitochondria are the organelles responsible for primary cellular ATP and ROS production, ensuring the

survival of cells by providing them with energy in the form of ATP and, under certain circumstances, to their destruction through their active participation in apoptosis. Mitochondria were shown to be crucial for the regulation of various physiological processes (150). Mitochondrial (mt) dysfunction is frequently observed in glioblastoma and has been linked to mt energy metabolism alterations, mt structure abnormalities, disturbances in mt membrane potential regulation, genomic mutations in mtDNA and apoptotic signaling, as well as to mutations involving the Krebs cycle enzyme isocitrate dehydrogenase (IDH) (148, 151). Mitochondria-targeted therapeutic strategies in glioblastoma include metabolic modulation with emphasis on dichloroacetate, a pyruvate dehydrogenase kinase (PDK) inhibitor (150, 152, 153) and mitochondrial-mediated apoptosis induced by tricyclic antidepressants (154), as well as mitochondrial aberrant signaling cascades with natural compounds such as phytosterol (148, 155). Mitochondria is also involved in the synthesis of cholesterol and 27-OHC, making it an interesting target for metabolic therapy.

Use of antisense therapies directed against the IGF-IR

The modification of the expression of growth factors or their receptors is implicated in tumor progression (156). The insulin-like growth factor type I receptor (IGF-IR) has been shown to contribute to the tumorigenesis process (157). IGF-I may also contribute to abnormalities of cholesterol metabolism (158, 159). IGF-I binding triggers the activation of several intracellular signaling cascades involving the mitogen-activated protein kinase (MAP-K) and the PI3K pathways (157). Inhibition of the expression or function of this receptor within tumor cells has been successfully achieved by different approaches, including the use of ribonucleic acid (RNA) or oligonucleotides antisense. Antisense RNAs and oligonucleotides inhibit the translation of messenger RNA (mRNA) (160, 161). These antisense approaches to control IGF-IR expression are indeed capable, in experimental models, of blocking the expression of the receptor in glioblastoma cells and inhibiting their tumorigenesis *in vivo* by inducing cellular apoptosis and/or an immune response (162, 163).

Targeted nanotherapy

Glioblastoma therapies are not fully effective due to the existence of a series of barriers that prevent them from reaching these tumors. Great hopes are placed in nanotherapy, since nano-drugs could improve the delivery of glioblastoma drugs (164). Nanotherapy could be used to address drugs specifically acting on cholesterol metabolism in glioblastoma cells. Moreover, if nanoparticles are magnetic or superparamagnetic, they may be guided in a magnetic field. Nanotherapy could increase the therapeutic effectiveness of chemotherapeutic agents while reducing their side effects and favoring their passage through the BBB (165). However, two drawbacks of nanotherapy should be stated: (i) the need to remove certain metals from the treatment area when using metal nanoparticles, such as iron oxide or gold nanoparticles, and (ii) the indefinite exclusion of magnetic resonance imaging (MRI) for subsequent diagnosis of tumor progression (166). Nanoparticles can accumulate specifically in cancer cells through two targeting mechanisms: either they target passive cancer tissues by extravasation of nanoparticles through the

increased permeability of endothelial cell junctions in the tumor, or they target the tumor cell by functionalizing the surface of the nanoparticles with ligands which specifically bind to receptors that are overexpressed at the cancer cell surface (167). Another possible treatment for glioblastoma patients could be intra-tumoral thermotherapy using magnetic iron-oxide nanoparticles combined with radiotherapy (168). Even a 7-KC-containing nano-emulsion could be of interest to treat glioblastoma since 7-KC has been successfully used to reduce melanoma growth (169).

CONCLUSION

Cholesterol derivatives, including oxysterols, that have anti-proliferative and immunosuppressive properties, could have a great potential for the treatment of cancer (170, 171). Furthermore, oxysterols modulate the activity of several proteins and consequently affect many cellular functions and influence various physiological processes including cholesterol metabolism by maintaining cellular cholesterol level (105). Moreover, oxysterols have been revealed to modulate the function of immune cells and cancer growth. These effects can be dependent on the activation of the oxysterol-binding LXRs (170). At micromolar concentrations, some oxysterols are cytotoxic towards cancer cells in culture, and reduce the growth of murine transplanted tumors (172). Thus, due to the important role of oxysterols in cancer, possible applications of cholesterol derivatives as immunosuppressants or as active anticancer agents in metabolic therapy are promising. Tt has been shown that several cholesterol derivatives, which may or may not be LXR-agonists, induce numerous organelle dysfunctions including mitochondria, lysosome, peroxisome and endoplasmic reticulum, and are also autophagic inducers, these molecules could thus be of interest in the treatment of glioblastoma by targeting their cancer cells' metabolism.

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7

Targeting Energy Metabolism to Overcome Therapeutic Resistance of Glioblastoma and Tumor-associated Edema

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Abstract: Glioblastoma remains among the most lethal of human malignancies. The current standard of care prolongs life expectancy about 2 months on average compared to from radiation therapy alone, leading to a median patient survival of 14.6 months. Glioblastoma is heterogenous tumor at various levels, and intrinsically resistance to radiation and chemotherapy. These limits therapeutic

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options for both primary and recurrent tumors. Importantly, glioblastoma progression is often accompanied by cerebral edema, a significant cause of morbidity that influences the clinical course and prognosis of the disease. Immunosuppressive corticosteroids have been the primary treatment for glioblastoma-associated edema. However, the effect is temporary and accompanied by adverse effects due to the action of corticosteroids outside of the targeted area. Research over the past two decades has unveiled a significant role for metabolic reprogramming that confers a survival advantage during gliomagenesis and therapeutic resistance. This chapter introduces the recent discoveries of two energy metabolism pathways: AMP-activated kinase-mediated stress-resilient glioblastoma growth, and Guanosine-5'-triphosphate (GTP)- metabolic reprogramming that renders anabolic growth and radioresistance. We discuss the potential clinical utility of currently available medicine that could target these metabolic pathways to suppress malignant growth of glioblastoma and increase the efficacy of the current glioblastoma therapy.

Keywords: energy metabolism; edema; purine nucleotide metabolism; radioresistance; radiosensitivity

INTRODUCTION

Gliomas are the most common malignant primary tumors of the central nervous system (1). Glioblastoma (GBM) is the grade IV glioma based on the WHO classification (2), and constitute about 54% of all gliomas (1). For high-grade gliomas (*i.e.*, WHO grade III and IV), the 5-year survival rate is below 10%, even with aggressive treatment of surgical resection with adjuvant radiation and chemotherapy. Even low-grade glioma (WHO grade II) are ultimately lethal, with a median survival term of 6–8 years (3, 4). Currently, curative treatments are unavailable for glioma.

Radiotherapy is one of the primary treatment modalities (5), constituting a part of the current standard of care (6). However, glioblastomas are intrinsically resistant to radiotherapy (7–15) due to increased ROS resistance mediated by mechanisms not currently understood (13, 14, 16–18). Radiation therapy yields only marginal improvements in patient survival (19, 20), with a recurrence rate of nearly 80% despite use of high dose radiation (21, 22). The current standard of care treatment for glioblastoma includes maximal safe surgical resection followed by adjuvant radiotherapy plus DNA alkylating reagent temozolomide chemotherapy, which prolonged a median patient survival of 14.6 months, from that of 10. 6 months of radiotherapy alone (19, 20).

Most glioblastoma patients (>60%) suffer from glioblastoma-associated cerebral edema that represents a major cause of morbidity in glioblastoma. Patients with cerebral edema experience headaches, seizures, dysphagia, and cognitive and personality changes. The accumulation of fluids increases intracranial pressure, leading to ischemia, herniation, and ultimately death (23). Furthermore, glioblastoma-associated edema influences the clinical course and the prognosis of the disease (24, 25). Inflammation and neoangiogenesis, which destroy the integrity of the blood-brain barrier (BBB) causing fluid leakage, are two major causes

of glioblastoma-associated edema. Immunosuppressive corticosteroids have been the primary treatment for glioblastoma-associated edema since the 1960s. However, the effect is temporary and accompanied by adverse effects due to the systemic effects of corticosteroids (26–28). Importantly, recent studies show that corticosteroids may reduce survival in human glioblastoma patients (26–28) and murine glioblastoma model (29). Vascular endothelial growth factor (VEGF)-signaling inhibitor bevacizumab (Avastin) has an anti-edema effect; however, it does not extend patient survival (30–32) and causes adverse events, including hypertension, arterial and venous thrombosis, intracerebral hemorrhage, and slow wound healing (30, 33–35). Our recent studies about energy metabolism in GBM implicate the potential of repurposing existing drugs that could lead to the resensitization of glioblastoma patients to radiation therapy or/and suppress glioblastoma-associated edema while inhibiting tumor growth.

In the past decades, extensive research has uncovered genetic mutations (36–43), transcriptional changes (44–51), and reconfiguration of signaling pathways (49, 52–55) in glioblastoma pathogenesis. These studies reveal that glioma is highly heterogeneous, enabling multiple robust transcriptional, signaling, and metabolic programs that mediate apoptosis resistance of glioblastoma during tumorigenesis and confer therapeutic resistance. Importantly, even before the era of molecular biology, metabolic changes in glioma have been noted (55, 56). In the 1940s, a series of biochemical analyses conducted on human brain tumors, including glioma, revealed significant elevations of lipids in these tumors, particularly glioma (56, 57). More recent studies with advanced molecular methods and high-sensitivity mass spectrometry-based analytical methods have clarified a mechanistic basis of the metabolic changes to increase lipid synthesis and accumulation of lipid droplets in glioma and glioma stem cells, contributing to the malignant growth of gliomas (58–61). The changes in nucleotide metabolism in glioma was denoted in the early 1950s (62), which is in part confirmed by enzymatic analysis that shows dramatic suppression of salvage GTP biosynthetic enzymes in glioma in 1994 (63), further followed by recent molecular studies (64, 65). These metabolic changes provide the building blocks for major cellular constituents—proteins, lipids, and nucleotides—to match the high metabolic demand of rapidly growing glioma cells (66). Notably, more recent studies, including ours, have shown critical metabolic pathways that induce coordinated anabolic growth through multiple mechanisms (67–70).

This chapter introduces two energy metabolism-related signaling pathways—AMP-activated kinase (AMPK) and guanosine-5'-triphosphate (GTP) metabolism—that are activated in glioma. Then, we discuss the possible therapeutic benefits of targeting these energy metabolisms to suppress glioma progression and sensitize glioma for the current therapeutics in particular radiotherapy.

EMERGING ROLES OF ENERGY METABOLISM IN GLIOBLASTOMA AND THERAPEUTIC TARGETING

In part, glioblastoma malignancy stems from its increased resistance to stress conditions during gliomagenesis, which is positively associated with therapeutic

resistance, including radiation therapy (7–15). Until recently, whether and how cellular metabolism is integrated into the process of glioma formation, progression and stress resilient growth is understudied. This section introduces emerging roles of energy metabolism in gliomagenesis and its potential clinical utility as a new therapeutic target for glioblastoma.

ATP energy sensor, AMPK, is critical to overcoming stresses during gliomagenesis

Stress is central to tumor evolution (71). The success of tumor cells in the hostile tumor milieu depends on how well tumor cells activate stress management pathways. Metabolic stress in solid tumors like glioblastoma poses a formidable challenge for tumor cell survival. These stresses include nutrient, hypoxic, pH, and oxidative stress in addition to therapy-induced xenobiotic stress (71–73). Metabolic stress is often caused by energy stress, which reduces the cellular ATP to AMP ratio and activates the energy sensor AMPK (74, 75) (Figure 1). Once activated, AMPK augments energy-generating reactions such as glycolysis and mitochondrial oxidative phosphorylation of glucose and fatty acids (74, 75).

Because AMPK is part of the liver kinase B1 (LKB1) tumor suppressor pathway and turns off major biosynthetic reactions such as lipid and protein synthesis—processes that are key to tumor cell growth and proliferation—it was long

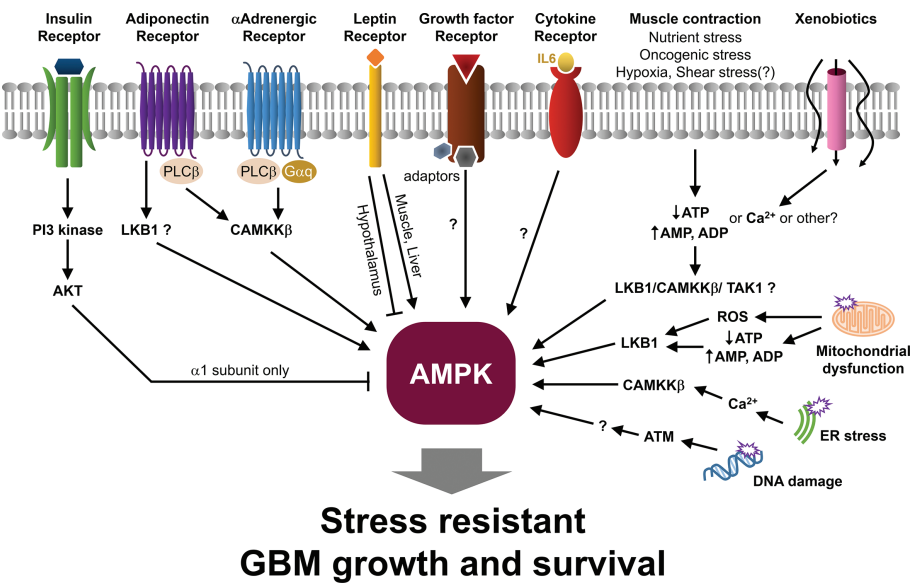

Figure 1. Activation of AMPK pathway has glioblastoma cells to be high stress resistant. AMPK receives many intra- and extra-cellular signals as a part of LKB1, CAMKKb, and other pathways. Activated AMPK leads to high stress resistance of GBM cells and supports their growth and survival.

believed that AMPK had a net suppressive role in tumorigenesis, including glioblastoma (76). However, AMPK-deficient transformed cells under tumor-like hypoxic conditions have a growth disadvantage *in vivo* (77). Taking an orthogonal approach, we determined that AMPK activity is abundant in all high-grade gliomas regardless of the genetic background of the tumors (78, 79). We showed that through transcriptional control of glioblastoma bioenergetics AMPK is required for optimal growth and survival of glioblastoma (79). Studies from other laboratories also concluded a role for AMPK in glioma pathogenesis. In an N-ethyl-N-nitrosourea-induced rat model of brain tumors, high AMPK activity was reported from the early hyperplastic lesions to the fully formed tumors (80). In a mouse model of astrocytoma driven by mutant *HRas* and *Pten* deletion, AMPK was necessary to maintain astrocytoma proliferation and survival (81) and lipoprotein internalization (82). The inhibitory role of AMPK on major biosynthetic processes that are required for cell growth and division appears paradoxical to the presence of high levels of active AMPK in glioblastoma and other solid tumors. However, as the tumor grows in volume, a plethora of tumor-specific stress builds up. This includes oncogenic stress, nutrient and oxygen stress due to fluctuating nutrients and oxygen levels and malformed neovasculature, and pH stress caused by the harsh acidic environment. These stresses reprogram tumor metabolism that allows tumor cells to survive and thrive in this stressful tumor microenvironment. Although the mechanisms are not fully clear, active AMPK may support this altered tumor metabolism and tumor cell survival (74).

A potential of AMPK targeting to enhance the efficacy of radiation therapy

One of the important consequences of AMPK activation is the upregulation of autophagy. Importantly, the enhanced autophagy contributes to radioresistance in glioblastoma and many other tumors (83–86). *A priori*, AMPK activation constitutes a key element of glioblastoma radioresistance (87, 88). Up to now, agents that indirectly activate AMPK were used to suppress tumor cell growth, including glioma growth (89–91). Notable agents include the antidiabetic biguanide drugs (metformin and phenformin) and the *de novo* purine synthesis pathway metabolite AICAR. Biguanides inhibit mitochondrial complex I and cause energy stress, while AICAR metabolizes to ZMP, which mimics AMP—each process activating AMPK (92, 93). Importantly, metformin has been shown to increase radiosensitivity (94–96). Although this may appear paradoxical, studies from our laboratory have shown that the anti-glioma effects of AICAR and biguanides are not only AMPK-independent but, in fact, AMPK-silenced glioma cells lose metabolic plasticity and become more vulnerable to the cytotoxic effects of AICAR and biguanides (78). This loss of metabolic plasticity of AMPK pathway deficient cells is likely conserved across other tumor types since LKB1 null lung cancer cells are also hypersensitive to biguanides (97). Together, results from preclinical and clinical studies illuminate a unique opportunity to use biguanides in clinical trials in combination with AMPK inhibitors, which are currently under development in our laboratory. The expectation is that this combination will likely synergize to overcome the radioresistance of glioblastoma.

GTP METABOLIC REPROGRAMMING PROMOTES GLIOBLASTOMA MALIGNANCY

ATP and GTP are involved in many cellular functions, including DNA and RNA building blocks, energy sources, enzymatic cofactors in metabolic pathways, and components of signal transduction. There are two pathways to produce GTP. *De novo* GTP synthesis involves a multi-step, high nutrient and energy-consuming pathway. Glucose is converted to GTP through 19 enzymatic steps that use a glycine molecule, an aspartate molecule, 3 glutamines, 2 N^{10}-formyl-THF, and 10 ATP. In contrast, the salvage pathway is an energy-efficient process in which a nucleoside (inosine, guanosine) and a nucleobase (hypoxanthine, guanine) are recycled to produce a GTP (98, 99) (Figure 2). As a result, the use of the salvage pathway is heavily favored in adult tissues, particularly in the adult brain (100–102). Importantly, many tumors increase GTP levels more than the other ribonucleotides, including glioblastoma (65, 103). However, how and why tumors alter GTP metabolism for their malignant growth has not previously been explored.

To that end, we have discovered a lipid kinase PI5P4Kβ as an intracellular GTP sensor regulating the metabolism and the tumorigenic process in accordance with cellular GTP energy levels (104, 105). Also, a recent publication of ours showed that GTP biosynthesis is significantly upregulated in glioblastoma by IMP dehydrogenase isozyme-2 (IMPDH2), which promotes enhanced ribosome biogenesis and tRNA synthesis, cooperating malignant glioblastoma growth *in vitro* and *in vivo* while normal brain cells operate without this GTP biosynthetic pathway (65). IMPDH2 mRNA expression is not significantly correlated with IMPDH2 protein levels, suggesting posttranscriptional regulation and therefore the importance of immunohistochemical analysis to evaluate the IMPDH2 levels. Importantly, increased IMPHD2 is correlated with poor survival of glioma patients regardless of IDH mutational status (65). Mechanistically, IMPDH2 upregulation promotes *de novo* GTP biosynthesis for ribosome biogenesis and tRNA synthesis, leading to nucleolar enlargement and malignant growth of glioblastoma (Figure 3). Inhibition of IMPDH2 decreases nucleolar size and significantly suppresses glioblastoma growth in *vitro* and *vivo* (65). The significance of IMPDH2 in glioblastoma and multiple cancers is also supported by other studies (68, 99, 106). Together, these studies illuminate the potential of targeting IMPDH-dependent GTP synthesis as a treatment for glioma. Importantly, there are FDA-approved inhibitors for IMPDH, including MPA and MMF (Figure 3) (106).

Targeting the GTP metabolic reprogramming to increase the efficacy of radiation therapy

Nucleotides are essential factors for genome stability and DNA repair (107–110). Importantly, studies of radioresistant bacteria, *Micrococcus luteus*, suggest that the GTP-metabolism is associated with radioresistance (111, 112). In cancer cells, IMPDH inhibition causes DNA lesions (113) and suppresses DNA damage-repair induced by radiation (114, 115). Radioresistant glioblastoma cell lines and glioblastoma-stem-like cells are capable of increasing guanylate levels in response to radiation (116). MPA/MMF treatment prevents this, leading to decreased DNA

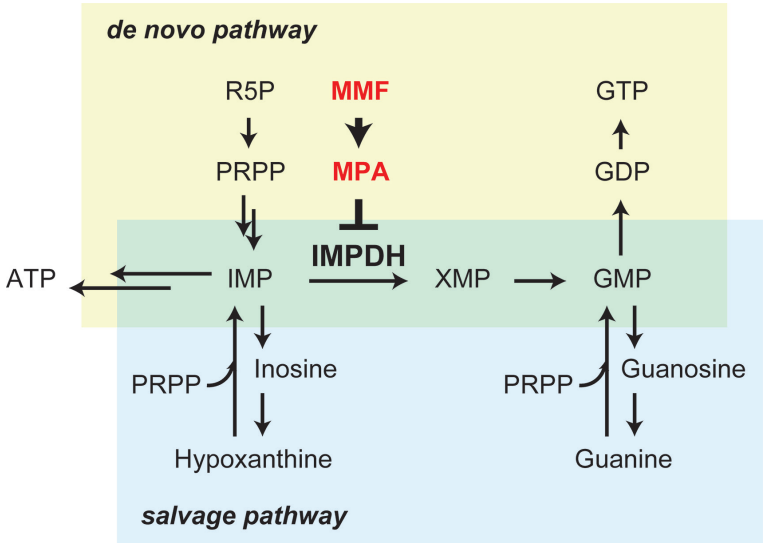

Figure 2. Two types of GTP synthesis pathways. GTP synthesis is controlled by two pathways. A sugar, phosphoribosyl diphosphate (PRPP), is made by ribose-5-phosphate (R5P) involved in pentose phosphate pathway. In *de novo* pathway, IMP is generated from PRPP through high nutrient and energy consuming reactions. On the other hand, salvage pathway produces a new IMP or GMP by directly connecting a sugar (PRPP) and a nucleobase (hypoxanthine or guanine). These nucleobases come from recycled IMP or GMP metabolites (inosine or guanosine). This economical pathway is favored in adult tissues. IMP dehydrogenase (IMPDH) oxidizes IMP to XMP. IMPDH is involved in the first step of guanine nucleotide synthesis and plays important roles in proliferation, cellular homeostasis, and also tumors facilitation including GBM. IMPDH activity is strongly inhibited by mycophenolate mofetil (MMF), which is a precursor of mycophenolic acid (MPA) and uses as prodrug of immunosuppressant. ATP, adenosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; IMP, inosine monophosphate; IMPDH, IMP dehydrogenase; MMF, mycophenolate mofetil; MPA, mycophenolic acid; PRPP, phosphoribosyl diphosphate; R5P, ribose-5-phosphate; XMP, xanthine monophosphate.

repair and clonogenic glioblastoma growth, thereby extending survival in an orthotopic PDX-glioblastoma model (116). In osteosarcoma U2OS cells, IMPDH2 overexpression increases radioresistance, while IMPDH2 knock-down increases radiosensitivity (117). These results suggest the previously unrecognized role of IMPDH2 in radioresistance (Figure 3). Importantly, Phase 0/1 Trial (NCT04477200) looking at the effects of MMF with radiation has been initiated to define the maximum tolerated dose of MMF when administered with radiation, in patients with recurrent glioblastoma or recurrent gliosarcoma. As of December 2020, our multidisciplinary group at the University of Cincinnati is in the preparation of a new MMF trial for glioblastoma treatment from a different angle, which is to treat GBM-associated edema by MMF. Collectively, repurposing IMPDH inhibitors has an important potential for new glioblastoma therapeutics and should be further studied to develop more effective, optimally designed therapeutics for clinical utilization to overcome radiation resistance and complications associated with glioblastoma.

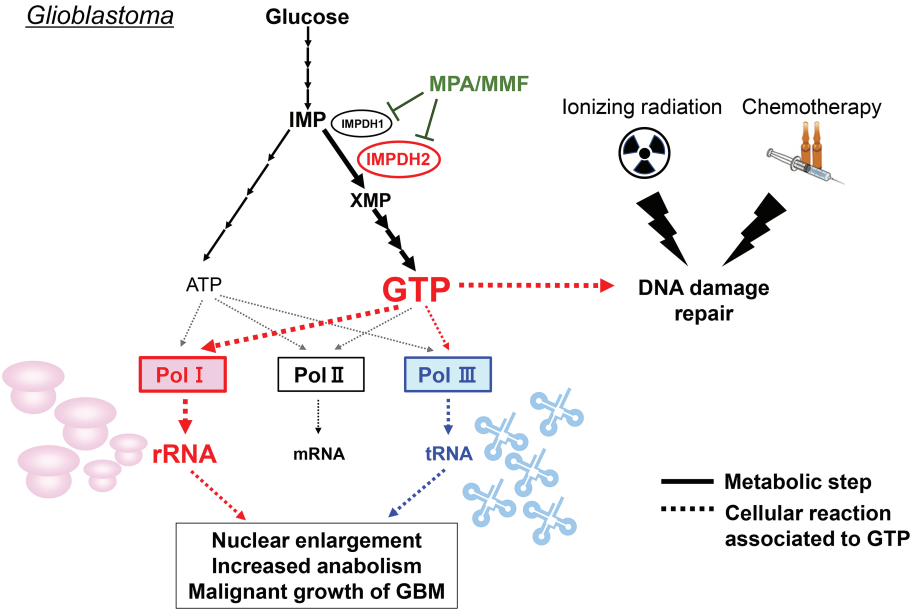

Figure 3. Upregulation of *de novo* GTP biosynthesis by IMPDH2 generates aberrant phenotype of Glioblastoma. In Glioblastoma (GBM), *de novo* GTP biosynthesis is upregulated by IMPDH2. Increasing GTP levels promotes rRNA and tRNA synthesis through transcription by RNA polymerase I (Pol I) and RNA polymerase III (Pol III) respectively. Upregulation of r/tRNA synthesis cause nuclear enlargement, increased anabolism, and malignant growth. Moreover, elevated GTP promotes DNA damage repair for radioresistance in GBM. MPA and MMF, the inhibitor for IMPDH, block both r/tRNA synthesis. Moreover, decrease in GTP levels by MPA and MMF indirectly inhibits GTP-associated DNA damage repair. Solid line indicates the metabolic step and dotted line indicates the cellular reactions associated to GTP.

A possible utility of the immunosuppressive effect of MMF to ameliorate glioblastoma-associated edema

Since MMF has been used as a potent immunosuppressor for tissue transplanted patients and autoimmune disease, a potential caveat of MMF or any IMPDH inhibitor is that it may limit the use of an upfront glioblastoma setting. However, we propose that MMF's use may be beneficial in some situations, particularly glioblastoma-associated edema treatment, based on the following evidence:

(i) *MMF suppresses inflammation and stroke-associated edema:* MMF is used globally for organ transplanted patients and possesses greater potency for the IMPDH2 isozyme (118). Importantly, MMF inhibits activation of microglia and astrocytes (119) and monocyte recruitment to endothelial cells (120, 121). In the LPS-stimulated BALB/c mouse neuroinflammation model, MMF treatment suppressed the expression of pro-inflammatory proteins (for example, iNOS, COX-2, TNFα, IL-1β, IL-6) (122). Furthermore, MMF treatment suppressed cerebral edema in stroke-prone spontaneous hypertensive rats (SHR-A3) (123).

(ii) *MMF suppresses neoangiogenesis:* Several reports indicate a critical link between IMPDH2 and neoangiogenesis. Two studies using zebrafish embryos show that IMPDH2, but not IMPDH1, is highly expressed at the sites of new blood vessels, and MPA treatment suppresses angiogenesis (124, 125). MPA treatment suppressed angiogenesis of human endothelial cells (126, 127). Oral administration of MMF significantly suppressed *in vivo* angiogenesis induced by melanoma (128), pancreatic cancer (129, 130) and U87MG glioma (127). Importantly, our preliminary studies using hCMEC/D3 cells, widely used as BBB models (131–137), show that MPA treatment does not disrupt the integrity of BBB.

Thus, MMF treatment has a high potential to suppress neoangiogenesis while maintaining BBB integrity. Currently, our multidisciplinary group at the University of Cincinnati is actively pursuing research to clarify the utility of MMF for glioblastoma edema treatment.

CONCLUSION

Despite the advances in general cancer treatment, glioblastoma remains among the most lethal of human malignancies. Even with aggressive multimodal radiation and chemotherapy after surgery, radiation therapy yielded marginal improvements in patient survival (19, 20) due to the radioresistant nature of glioblastoma. It is crucial to develop more effective therapeutics to improve the prognosis of the average patient with a glioblastoma and identify glioblastoma vulnerabilities for new potential targets and test the setting in clinically relevant glioblastoma animal models. In this chapter, we have introduced new potential targets for glioblastoma therapy, which are expected to suppress glioblastoma regardless of mutational status and increase the efficacy of the current therapeutic regimen when combined. For the next stage, it is crucial to further investigate the drugs targeting AMPK and IMPDH on the survival, therapeutic resistance, and edema formation of immunocompetent glioblastoma mouse models, and assess pharmacodynamics and identify PD markers. It is also imperative to study the combinations of these drugs with radiation therapy, including upfront proton beam therapy as introduced in the following chapter.

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Therapeutic Potential of Curcumin for the Treatment of Malignant Gliomas

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Abstract: Glioblastoma is the most common primary malignancy of the central nervous system. Maximal surgical resection of glioblastoma in addition to temozolomide and fractionated radiation therapy provides an overall median survival of approximately 15 months. The addition of tumor-treating fields (Optune therapy) has the potential to increase median survival to 20 months, although compliance and ease of use remain an issue. Glioblastoma remains a devastating diagnosis fraught with complications. Curcumin is a yellow pigment from the rhizome of the ubiquitous and commercially available spice, turmeric (*Curcuma longa*). Turmeric has been long used in Indian traditional medicines and has been established as a safe food additive by the US Food and Drug Administration. There is a wealth of *in vitro* data suggesting that turmeric's main active component, curcumin, has many favorable effects on glioblastoma. Curcumin has been shown to potentiate the effects of chemotherapy and radiation, decrease malignant spread, protect normal tissue from oxidative stress, and regulate many genetic targets resulting in glioblastoma cell death. Curcumin's positive safety profile and potential therapeutic effects on glioblastoma make it a promising potential adjunct to current standard treatment regimens.

Keywords: anti-glioma activity; central nervous system tumors; curcumin; glioblastoma; high-grade gliomas

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INTRODUCTION

Gliomas have historically been divided into 4 grades by WHO classification, with highly variable prognosis between the histologic grades. WHO grade I gliomas include pilocytic astrocytoma, subependymoma, subependymal giant cell astrocytoma, ganglioglioma, desmoplastic infantile astrocytoma, and myxopapillary ependymoma, among others. WHO grade II gliomas are infiltrative and generally more aggressive than grade I tumors and include oligodendroglioma, fibrillary astrocytoma, pleomorphic xanthoastrocytoma, and mixed oligoastrocytoma, as well as other less common tumor types. WHO grade III gliomas include anaplastic astrocytoma, anaplastic oligoastrocytoma, anaplastic ganglioglioma, anaplastic ependymoma, and anaplastic pleomorphic xanthoastrocytoma. Finally, WHO grade IV gliomas include glioblastoma, various subtypes of glioblastoma, and pinealoblastoma (1). Within the category of diffuse, infiltrating gliomas (WHO II, III, IV), tissue diagnosis was traditionally made based on histopathologic analysis based on the presence or absence of microvascular proliferation, necrosis, and mitotic activity. The introduction of genetic profiling of gliomas based on IDH mutation, MGMT promoter methylation status, 1p/19q codeletion has led to better understanding and more appropriate classification of gliomas and their subtypes (1).

With an annual incidence of 3.21 per 100,000 individuals, glioblastomas are the most common primary malignant brain tumor with nearly 11,000 new cases diagnosed each year in the United States (2). According to the CBTRUS data repository, glioblastoma accounted for 14.5% of all CNS tumors, 57.7% of all gliomas, and 48.6% of all malignant CNS tumors diagnosed from 2013–2017 (2). Prognosis for patients with glioblastoma remains dismal. Aggressive maximal resection followed by radiotherapy has a median overall survival of 12.1 months. The addition of adjuvant alkylating chemotherapy with temozolomide (TMZ) and radiotherapy after surgery (Stupp protocol) increased median overall survival time to 14.6 months (3). Optune® therapy (Novocure Inc., Haifa, Israel) is a wearable technology that delivers low intensity alternating electrical fields over the scalp. Tumor-treating fields along with maximal surgical resection and Stupp regimen increases median overall survival time to 20.9 months, although it has not been used clinically in a widespread manner (4). It is important to note that median overall survival for all cases of glioblastoma is around 8 months (2).

Given the poor prognosis for patients with newly diagnosed glioblastoma, it is imperative that efforts for drug development continue in order to improve upon current standard-of-care therapies. Curcumin, a yellow pigment, derives from the rhizome of the ubiquitous and commercially available spice, turmeric (*Curcuma longa*). Turmeric has been long used in traditional Indian Ayurvedic medicine, with its medicinal use dating back to 2500 years ago, and more recently for its anti-inflammatory properties (5). Curcumin has also been studied for its apparent anti-tumor effects systemically. More recently, Curcumin has become an interesting consideration for glioblastoma treatment because of its modulation of multiple targets which appear to suppress tumors, decrease malignant characteristics, promote apoptosis, and potentiate the effects of chemotherapy and radiation (6–9). Interestingly, the number of peer-reviewed publications related to curcumin has been steadily increasing over the past 20 years, with an expanding portfolio of scientific reports pertaining to curcumin and gliomas.

CHEMICAL AND PHYSICAL PROPERTIES OF CURCUMIN

Curcumin ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is one of three phytoconstituents (non-nutrient bioactive compound) of turmeric; the other two phytoconstituents are bis(demethoxy)curcumin and demethoxycurcumin (Figure 1). Together, these compounds account for under 10% of turmeric's dry weight (10, 11). After removing the protein, carbohydrates, fats, minerals, volatile oils, and fiber from raw turmeric, a crude curcuminoid extract is generated, consisting of 60–70% curcumin, 20–27% demethoxycurcumin, and 10–15% bis(demethoxy)curcumin.

The molecular weight of curcumin is 368.38 daltons. Curcumin's β-ketone moiety exhibits keto-enol tautomerization, with the enol form predominating in alkaline solution. It is essentially insoluble in water at room temperature and has a neutral pH. Curcumin is photoactive and absorbs light wavelengths in the visible range of 408–500 nm and exhibits photodegradation. At 37 °C the half-life at pH 7.2 is less than 10 minutes; *in vitro* degradation of curcumin occurs via nucleophilic substitution or elimination by solvent molecules (solvolysis), and oxidative degradation (10).

In vivo studies indicate that the bioavailability of unaltered curcumin preparations (standard curcumin) is very poor; 12 g oral doses of curcumin administered to healthy human subjects produced serum concentrations of 50.5 ng/ml, although other studies saw increased concentrations with concomitant administration of piperine–the alkaloid responsible for the pungency of black pepper (*Piper nigrum*)–indicating that bioavailability concerns can be at least partially addressed (12, 13). For orally ingested doses, the bioavailability issue seems to start with intestinal mucosa via modification by glucuronidation and sulfonation. Orally administered curcumin doses of 3.6 g are detected in colorectal tissue; however, in other studies utilizing this dose range, the serum concentration was nearly undetectable (12, 14). During phase I metabolism, oxidoreductases contribute to the reductive metabolism of curcumin. In phase II metabolism, intestinal epithelial cells' glucuronosyltransferases and sulfotransferases act by glucuronidation and sulfonation of curcumin. The majority of curcumin that reaches systemic circulation is reduced, conjugated, and excreted in feces, not exhibiting pharmacodynamic effects outside of the alimentary system (15).

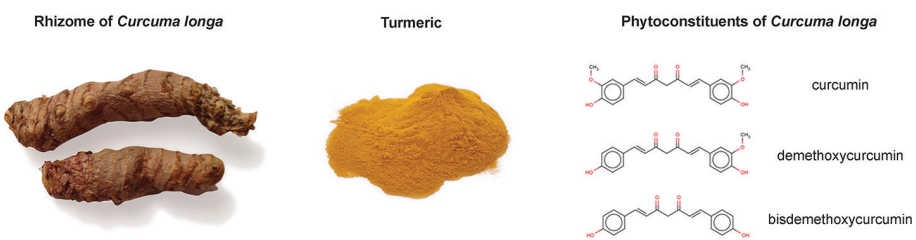

Figure 1. Curcuminoids extracted from the rhizome of turmeric (*Curcuma longa*) and molecular structures of the three phytoconstituents: curcumin, demethoxycurcumin, and bisdemethoxycurcumin.

TOXICITY

Curcumin is a very well tolerated substance. Daily intake of curcumin can approach 3 mg/kg/day; side effects in people ingesting 500–12,000 mg doses included headache, rash, and yellow stool (16). Multiple studies regarding the safety of orally ingested curcumin have been conducted. A phase 1 trial with 25 participants ingesting 8,000 mg curcumin per day for three months demonstrated no toxicity; five other studies using smaller doses (1,125–2,500 mg) also demonstrated curcumin's safety (17). If toxicity does occur, it is likely via curcumin's extensive interaction of hERG (human Ether-à-go-go-related gene) channels, cytochrome P450, or drug-drug interactions. In rat models, inhibition of cytochrome P450 has caused cardiotoxicity, while interaction with glutathione S-transferase has caused drug-drug interactions (10). Curcumin has been shown to induce cytotoxicity in human lymphocytes and renal cell lines at IC50 concentrations of 15.2 μM and 31 μM, respectively (10). Currently, the FDA has not approved curcumin as a treatment for any condition and it remains classified as a safe food additive at levels from 0.5 to 100 mg per 100 g of food.

CURCUMIN AND SIGNAL CASCADE PROTEINS

Curcumin appears to modulate many cellular processes, allowing it to act as an *in vitro* tumor suppressor, decrease malignant characteristics, promote apoptosis, and potentiate the effects of chemotherapy and radiation. Curcumin has been shown to interact with Wnt, HDGF, STAT3, and NRF2, which will be discussed in detail below.

Curcumin and BIRC5

Baculoviral inhibitor of apoptosis (BIRC5), or survivin, is a protein expressed in embryonal tissues. As a member of the inhibitor of apoptosis (IAP) family of proteins, BIRC5 is a G2/M cell cycle regulator, minimally expressed in mature tissue (18, 19). BIRC5 overexpression has been implicated in protection from apoptosis and regulation of mitosis, and has been associated with worse outcome in renal cell carcinoma, esophageal cancer, and breast cancer (19). In a recent study, whole genome sequencing was performed in 144 patients diagnosed with glioblastoma; those found to have high BIRC5 expression had a worse prognosis than patients with low BIRC5 expression (20). One large retrospective analysis of 1,260 patients with gliomas suggested that BIRC5 expression was correlated with worse overall survival (19). Commercially available U87, U51, and U235 human glioblastoma cell lines along with additional patient-derived glioblastoma cells were treated with 25 μM curcumin solution. BIRC5 and IAP2 expression was found to be decreased after curcumin exposure at 1 and 6 hours. This effect is secondary to increased phosphorylated ERK, p38 and JNK; phosphorylated ERK inhibits STAT3, rendering it unable to translocate into the nucleus, therefore decreasing expression of BIRC5 and IAP2 (7).

Curcumin and RANK

Receptor activator of NF-κB (RANK), via its interaction with RANKL, activates survival signaling cascades though AKT and EGFR. Alternatively, RANK can promote apoptosis via c-jun N-terminal kinase activation, also known as tumor necrosis factor receptor superfamily 11A (21). U87 and U251 glioblastoma cells exposed to 15 μM and 30 μM curcumin solution had a respective 1.5–2.6-fold and 1.7–3.7-fold increase in RANK mRNA levels, accomplished through inhibition of STAT3. Separately, siRNA-specific knockdown of STAT3 also produced increased RANK expression (21).

Curcumin and Wnt

The Wnt signaling pathway is a critical regulator of brain development. Presynaptic and postsynaptic transcription is regulated by Wnt; its dysfunction has been linked with development of glioblastoma (22). The Wnt signal cascade begins on the surface of cells after the bunding of the frizzled/low density lipoprotein receptor-related protein complex. A complex cascade involving Dishevelled, GSK-3β, APC, and Axin ensues, with a subsequent intranuclear increase in β-catenin (23). The ultimate downstream effect of Wnt signaling is cell fate specification, differentiation, and mitogenic stimulation; aberrant Wnt signaling has been associated with development of glioblastoma (23). The Wnt/β-catenin pathway contributes to cell proliferation and tumorigenesis. U373 glioblastoma cells exposed to nano-micelle curcumin preparations had suppressed Wnt, as well as NF-κB activity with an overall effect of inhibited cell growth, tumor shrinkage, and decreased invasiveness (24).

Hepatoma-derived growth factor (HDGF) is a growth factor that interacts with β-catenin to promote tumor generation, progression, and metastasis; HDGF is also upregulated in gliomas (25). The HDGF/β-catenin complex was indirectly inhibited via inhibition of HDGF in one series with U251 and LN229 glioblastoma cells that were exposed to curcumin concentrations from 5 to 200 μmol/L, once again resulting in reduced proliferation, invasion and extent of tumor cell migration (25).

Curcumin and NRF2

Curcumin has been reported to exhibit a radioprotective effect in normal tissues, while sensitizing tumor cells to the effects of ionizing radiation (26). One possible mechanism for this phenomenon is curcumin's interaction with nuclear factor erythroid 2 related factor 2 (NRF2). NRF2 is a leucine zipper protein that is responsible for regulation of oxidative stress; it is normally bound to KEAP1, in an inactive state. Upon exposure to insults such as toxins or radiation, NRF2 dissociates from KEAP1 and accumulates within the cell's nucleus, ultimately resulting in antioxidant gene activation and resistance to oxidative stress (27). This is an important effect and suggests that curcumin may play a protective role in normal tissues that also receive systemic treatment or ionizing radiation. Curcumin is a known activator of NRF2 (27). Interestingly, NRF2 is upregulated in glioblastoma and is possibly responsible for glioblastoma survival in an environment under

increased oxidative stress (27). Not surprisingly, U87MG glioblastoma cells treated with NRF2 had decreased levels of oxidative stress proteins, decreased proliferative capacity, and decreased self-renewal compared to controls without NRF2 knockdown.

Curcumin and Protein Ubiquitination

Protein ubiquitination is an essential process involved in regulation of many signaling cascades; ubiquitination of NEDD4 results in protein degradation (8). NEDD4 has been implicated in the development of cancers and neurodegenerative diseases and exerts an oncogenic effect via dysfunctional ubiquitin signaling (28). E3 ubiquitin ligase is a key element of the ubiquitin pathway; its aberrant function has been shown to be involved in number of malignancies (29). Neural precursor cell expressed developmentally downregulated protein 4 (NEDD4) is an important member of the E3 ubiquitin ligase family that functions in substrate recognition for the ubiquitin pathway. Activation interaction with PTEN degradation via the ubiquitin cascade eventually activates the PI3K/AKT pathway, resulting in cellular proliferation and oncogenesis (30). SNB190 and A1027 human glioblastoma cells treated with 15 μM curcumin solution for 72 hours had diminished expression of NEDD4 on western blot and qPCR analysis, with reduced overall proliferative capacity (8).

S-phase kinase protein 2 (Skp2) is a component of the SKP-Cullin-F box complex, which facilitates the ubiquitin-mediated degradation of G1 checkpoint inhibitors G21, P21 and P27; the net effect is cellular proliferation via cell cycle progression (31, 32). Taking this into consideration, Skp2 overexpression results in unchecked cell cycle progression and has been associated with tumorigenesis, including gliomas (33–35). U251 and SNB19 glioblastoma cells treated with variable curcumin solutions demonstrated decreased Skp2 expression on RT-PCR. In line with the expected effect of decreased Skp2 expression, this study demonstrated concomitant decreases in migration, invasion, and proliferation (36). Part of curcumin's *in vitro* effects likely occur through modulation of protein ubiquitination, an incredibly complex and highly regulated process.

CURCUMIN DECREASES TUMOR INVASIVENESS

The malignant nature of gliomas is often due to the fact that tumor cells infiltrate deep into the parenchyma, distal to observable tumor margins; this makes complete surgical resection impossible (37). Biopsy studies in glioblastoma patients have shown that tumor cells can be found well beyond the MRI T2 hyperintense margins (38). Glioma invasion is promoted by the expression of matrix metalloproteinases (MMPs). MMPs are zinc-dependent proteolytic endopeptidases that degrade extracellular matrix proteins and cell adhesion molecules, allowing tumor cells to become locally invasive (39). Through a complex interaction with vascular endothelial growth factor (VEGF) and its receptor, VEGFR2, MMP-2 has been implicated in vasculogenesis, angiogenesis and disseminated tumor growth (39, 40). MMP-9 may promote vasculogenesis in the absence of VEGF (40). Large diameter gliomas appear to have greater expression of MMP-2 than their smaller

counterparts, with an accompanying decrease in expression of their inhibitor proteins (40). In one study, U373 glioblastoma cells treated with curcumin solution had measurable decreases in multiple MMPs including MMP-2 (41). Another study specifically examining invasion distance of SNB19 and A1027 glioblastoma cells after treatment with 10 µM, 15 µM and 20 µM curcumin solutions found that treated cell lines had significantly less migration (invasion) than controls (8). Increased expression of MMPs may very well contribute to the invasive properties of high-grade gliomas and curcumin may be an interesting drug to combat this characteristic.

CURCUMIN INDUCES OXIDATIVE STRESS AND APOPTOSIS IN GLIOMA CELL LINES

Curcumin has been shown to have cytotoxic effects in cancer cells via the production of reactive oxygen species (ROS). U87, U51, and U235 glioblastoma lines treated with 70 µM curcumin solution saw decreases in cellular viability to levels as low as 20%. At curcumin concentrations as low as 25 µM, immunofluorescence oxidative stress assays demonstrated induction of ROS at 1 and 6 hours, normalizing after 1 day (7). U373 glioblastoma cells treated with curcumin solution demonstrated preferential induction of apoptosis at concentrations of 10 µg/ml and similar levels of apoptosis and necrosis and curcumin concentrations of 20 µg/ml (41). This is contrary to the protective effect that curcumin exhibits against oxidative stress in normal tissue (26). This dual mode of action exerted by curcumin may be due to its ability to reduce oxidative stress and inflammatory response in normal cells while also having the ability to upregulate genes responsible for cell death in pathological conditions.

BARRIERS TO CURCUMIN AS A SYSTEMIC THERAPY

Despite curcumin's apparent ability to reduce the malignant nature of glioblastoma *in vitro* via multiple mechanisms, it has failed to gain traction as a therapeutic agent. This, in part, may be due to curcumin's poor systemic bioavailability. *In vivo* bioavailability studies indicate that the bioavailability of unaltered curcumin preparations (standard curcumin) is dismal. After all, a therapeutic agent is not useful if it cannot be effectively delivered to its target in sufficient quantities. There is evidence to suggest that curcumin crosses the blood-brain barrier and may offer some neuroprotective effects, especially in context of neurodegenerative diseases such as Alzheimer's disease. In a murine model, curcumin was shown to decrease beta-amyloid plaque burden by 43% via blockage of beta amyloid self-assembly (42, 43). Similarly, curcumin's anti-inflammatory properties, anti-oxidant properties, metal chelation properties, and ability to decrease lipid peroxidation and lipofuscin accumulation are thought to be neuroprotective (42). The extent or mechanism of transport of curcumin across the blood-brain barrier has not been well described in humans. Common explanations in favor of curcumin's transport across the blood-brain barrier reference its lipophilic nature. However, critics are

quick to mention that other large lipophilic molecules, such as cholesterol, cannot readily cross the blood-brain barrier. Specific transport mechanisms have not been described to date.

One of the main areas of focus for drug development of curcumin over the past years has been on preparations that increase its bioavailability. Factors that contribute to curcumin's low systemic bioavailability include its large crystalline structure, low solubility in non-toxic solution, and extensive *in vivo* metabolism (15, 44). Low-crystallinity nanoparticles were formed by exposing curcumin preparations to solvents such as chloroform. Although these preparations increased bioavailability of curcumin, their toxic solvents limited use in humans. The advent of precipitation by pressure reduction of gas-expanded liquid (PPREGL) has enabled the production of spherical curcumin nanoparticles as small as 66 nm (44). PPREGL nanocurcumin preparations utilize carbon dioxide instead of other volatile solvents to dry and precipitate curcumin nanoparticles from a curcumin impregnated nanoporous starch aerogel (NSA). The bioavailability of curcumin NSA nanoparticles are 173 times greater than that of standard curcumin preparations (0.4% vs 69.1%) (44).

Other methods of curcumin preparation that increase bioavailability include liposomal curcumin and curcumin micelles. Curcumin micelles have 19-fold greater bioavailability compared to standard curcumin preparations (45). Unfortunately, micelle preparations have the drawback of a relatively large volume of stabilizers and excipients that may cause side effects such as hemolysis (46). Liposomal curcumin preparations are a promising established method of increasing curcumin bioavailability, with the ability to affix transport molecules to enhance transport across the blood-brain barrier. The p-Aminophenyl-α-D-mannopyranoside transport molecule enhances liposomal transport across the blood-brain barrier and selectively targets cortex, hippocampus, cerebellum, brainstem, and pontine nuclei (47).

CONCLUSION

There is a dire need for advancement in glioblastoma therapy. Standard-of-care chemotherapy with temozolomide and radiation has not changed for over a decade and the prognosis for glioblastoma is dismal. Curcumin has a positive safety profile and its interaction with cell cycle regulators and regulators of oxidative stress make it an attractive possible adjunct to current standard chemotherapeutic agents and radiation in the treatment of glioblastoma. *In vitro* studies have demonstrated that curcumin potentiates the effects of chemotherapy and radiation on glioblastoma cells, while decreasing the ability of glioblastoma to proliferate, migrate, and recover from oxidative stress. Curcumin's main barrier to its potential use as a systemic agent is its poor bioavailability, limited by extensive modification by enterocytes and rapid metabolism. Recent progress has been made in enhancing curcumin's bioavailability by production of non-toxic nanopreparations; however, they have yet to make their way into large studies or clinical trials. While there is limited *in vivo* data, *in vitro* data suggests that curcumin can be a safe and effective anti-glioblastoma agent. Additional high-quality studies regarding the safety and efficacy of high bioavailability curcumin

preparations will be necessary to further elucidate the potential role of curcumin in glioblastoma treatment.

Conflict of Interest: The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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9

Survival Prediction in Gliomas: Current State and Novel Approaches

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Abstract: Gliomas are neurologically devastating tumors with generally poor outcomes. Traditionally, survival prediction in glioma is studied from clinical features using statistical approaches. With the rapid development of artificial intelligence approaches encompassing machine learning and deep learning, there has been a keen interest among researchers to apply these methods to survival prediction in glioma allowing for integrated processes that encompass pathology, histology, molecular, imaging, and clinical features. This chapter provides an overview of the emerging computational approaches that have the potential to revolutionize survival prediction in glioma. Machine learning and deep learning techniques, including support vector machine, random forest, convolutional neural network, and radiomics, are discussed.

Keywords: convolutional neural network; machine learning approaches; support vector machine; radiomics; survival prediction in glioma

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INTRODUCTION

Outcome prediction in glioma is of tremendous importance as it has the potential to aid in optimal patient management and patient counselling. In the past, outcome prediction has centered mainly on clinical features such as age and performance status, surgical features such as resection status, and pathological features such as morphology and Word Health Organization (WHO) grade. Molecular classification and identification of predictive and prognostic factors are now also considered (1–4). Traditionally, outcome prediction is carried out through recursive partitioning analysis (RPA) (3, 4). Glioblastoma patient survival by prognostic grouping was published initially in 1993 and employed RPA, a non-parametric statistical technique creating prognostic groups based on clinical features (3). It divided patients into six prognostic classes (I – VI) (3), later simplified to three classes (III, IV, and V/VI) and eventually revised to include only glioblastoma (4). Limitations included the lack of temozolomide-based stratification and molecular features (for example, O^6-Methylguanine-DNA-methyl transferase [MGMT] methylation), both rectified recently (1, 2).

In the clinic, the most debated scenarios regarding management and outcomes center on the elderly (5–10), patients with lower grade gliomas (11–14), and the administration of systemic therapy (for example, PCV [Procarbazine, CCNU and vincristine] *vs*. temozolomide, current *vs*. sequential, and number of cycles) (15–17). With respect to glioma in the elderly, a number of metrics including age (6), temporal muscle thickness (7) and geriatric assessment (8) have been employed to predict survival. Straube *et al*. created a scoring system incorporating age, performance status, MGMT status, the extent of resection, and aphasia and motor dysfunction after surgery, all of which were found to be associated with survival (10). In lower grade gliomas, current practice is based on old pathological classification of glioma (12). This has resulted in the lack of a consensus regarding optimal use of radiotherapy in patients with low grade glioma (LGG) (13), timing and dose of radiation, and timing of chemotherapy (14). For survival prediction, consensus is generally achieved by multidisciplinary review of histology, molecular and imaging factors. Gittleman *et al*. employed TCGA (The Cancer Genome Atlas) data to develop and externally validate a survival nomogram, which is available as a free online software, for lower-grade glioma patients. The final nomogram included factors that increased the probability of survival: grade II tumor, younger age at diagnosis, a higher KPS (Karnosfsky Performance Status) and IDH (isocitrate dehydrogenase) mutation (16). In high-grade glioma (HGG) including glioblastoma, there is ongoing controversy regarding the number of cycles of chemotherapy to be administered (15–17). Gittleman *et al*. developed a nomogram using the Cox proportional hazards (CPH) model, incorporating factors that increased the probability of shorter survival: age at diagnosis, male gender, lower KPS, subtotal resection, and unmethylated MGMT status. The nomogram assesses survival probabilities (6-, 12-, and 24-mo) for patients with newly diagnosed glioblastoma and can be used to counsel patients regarding treatment decisions and optimizing duration of treatment and, like LGG counterpart, is available as free online software (16).

NOVEL APPROACHES TO SURVIVAL PREDICTION IN GLIOMA

Novel approaches employ computational methods to generate survival prediction (Figure 1). The number of publications that analyze the relationship between clinical, pathological, histological and imaging factors, and survival in glioma have been increasing at a rapid pace (Figure 1). More than 50% of publications involving computational approaches to survival prediction in glioma were generated in the last two years with more than 46 manuscripts dedicated to this subject (Figure 1A). Most of the manuscripts have focused on correlating imaging findings to survival prediction, followed closely by molecular characterization, genetics, and digital pathology (Figure 1B). Most data employed in novel computational approaches originated from smaller single-institution data sets with the remainder employing TCGA, Chinese Glioma Genome Atlas (CGGA) and Multimodal Brain Tumor Image Segmentation (BraTS) (Figure 1C). Only a small percentage of reports employed Surveillance, Epidemiology, and End Results (SEER) data (Figure 1C) presumably secondary to limitations currently inherent in nonspatial

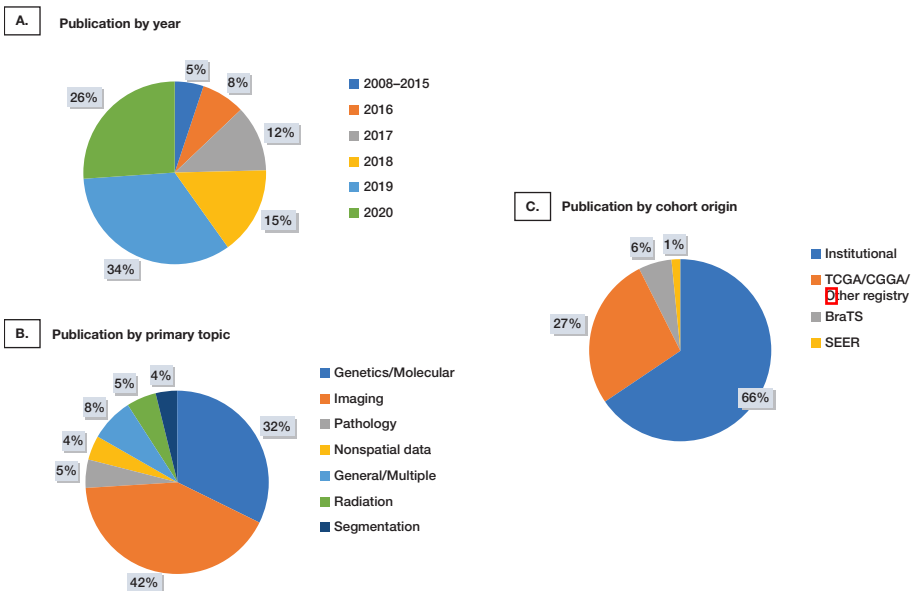

Figure 1. The landscape of computational approaches in the literature as related to glioma survival prediction. A. The % of publications related to glioma survival prediction by year of publication. **B.** The % of publications related to glioma survival prediction grouped by the primary topic explored (as defined by the primary data type employed in the prediction analysis). **C.** The % of publications related to glioma survival prediction grouped by the origin of the data employed to develop the approach.

data sets including the lack of capture of molecular and pathological features, and management. Almost exclusively, the data currently employed in glioma survival prediction is retrospective in nature. Prospective data is being generated in a small cohort of ongoing clinical trials with robust histopathology, molecular, and imaging endpoints but may yet take some time to be incorporated in ongoing computational approaches (Tables 1 and 2). Computational approaches generally involve subcategories under the umbrella of artificial intelligence namely machine learning (ML) and deep learning (DL) (Figure 2).

Machine learning approaches

With increasing accessibility of electronic health records and large-scale registry data, ML has become an increasingly popular method to model survival. ML is a sub-field of AI where a computer algorithm automatically develops a model that transforms input data to output without using rules defined by humans. Classical ML methods require input data to have well defined sets of variables in the format of structured data (features). The process of extracting relevant structured variables from raw data to be used as ML input, known as feature engineering, often requires significant domain expertise and computational processing power, especially when it comes to input data such as images and natural language. DL is an emerging sub-field of ML where the DL algorithm can take raw data, such as images, as input and "learn" to define its own features needed for computing

TABLE 1 Statistical analysis and machine learning terms employed in survival prediction

Term	Description
COX/CPH model	Cox's proportional hazards model: a regression model commonly investigating the association between the survival time of patients and one or more predictor variables (18).
RPA	Recursive Partitioning Analysis: a non-parametric statistical technique used to create prognostic groups based on clinical features (1).
C-index	Concordance index: A performance metric for how well a model predicts the ordering the patients' risk of death in comparison to ordering of patients' recorded survival time (19).
ROC	Receiver operating characteristics curve: A probability curve of sensitivity vs (1-specificity) when using different cutoff points in classifying binary outcomes (19).
AUC	Area under the ROC curve: Area under the ROC that is used distinguishes the discriminative potential of the algorithm (19).
Model validation	A ML trained model is evaluated with a test data set that is not used in training. A popular validation method is K-fold validation where the algorithm is trained K times with (1/K) of all data left out of training each time to be used to evaluate model performance (20).

TABLE 2 **Ongoing prospective trials with computational glioma survival prediction endpoints**

Trial	Data to be collected	Description/Goal	Endpoint(s)	Sponsor
NCT04215211: MR Based Survival Prediction of Glioma Patients Using Artificial Intelligence (21)	clinical, molecular, multimodal radiologic data (T1, T1c, T2, FLAIR, ADC, DTI, PWI), and detailed survival data	Create a registry containing clinical, molecular, multimodal radiologic data and detailed survival data of patients with primary gliomas. This registry will seek to construct and refine algorithms that are able to predict patients' survival in the frame of molecular pathology or subgroups of gliomas.	Primary: AUC of survival prediction performance	The First Affiliated Hospital of Zhengzhou University, China
NCT04215224: Histopathology Images Based Survival Prediction of Glioma Patients Using Artificial Intelligence (22)	clinical, molecular, histopathology imaging	Create a registry containing clinical, molecular, histopathology imaging data and detailed survival data of patients with primary gliomas. This registry will seek to construct and refine histopathology-based algorithms to predict patients' survival molecular pathology or subgroups of gliomas.	Primary: AUC of survival prediction performance	The First Affiliated Hospital of Zhengzhou University, China
NCT00330109: Identification of Clinically Occult Glioma Cells and Characterization of Glioma Behavior Through Machine Learning Analysis of Advanced Imaging Technology (23)	database of images (obtained using various advanced imaging technologies: MRI, MRS, DTI, and MET-PET)	Predict the locations of occult glioma cells, by learning the growth patterns exhibited by gliomas in previous patients by developing software tools that find gliomas similar to a current one, and that can autonomously find the tumor region within a brain image.	Primary: 1. Multimodal imaging 2. create an image-based database Secondary: Create ML algorithm to automate tumor segmentation, predict tumor behavior and predict location of clinically occult glioma cells	AHS Cancer Control Alberta, Canada

Table continued on following page

TABLE 2 **Ongoing prospective trials with computational glioma survival prediction endpoints (Continued)**

Trial	Data to be collected	Description/Goal	Endpoint(s)	Sponsor
NCT04216550: Prediction of Therapeutic Response of Apatinib in Recurrent Gliomas (24)	MRI and histology images	By leveraging artificial intelligence, this study seeks to construct and refine MR and histopathology imaging to predict the responses to Apatinib of patients with recurrent gliomas.	Primary: Changes of Response to Treatment Secondary: 1.PFS 2.OS 3.Adverse events	The First Affiliated Hospital of Zhengzhou University, China
NCT04359745: Improving Treatment of Glioblastoma: Distinguishing Progression From Pseudoprogression (25)	MRI images	Initial training, testing and cross validation of a classification model will be carried out using MRI data of glioblastoma obtained from publicly accessible imaging archives and King's College Hospital (KCH), London. For clinical validation, the trained model will undergo testing using MRI data from patients recruited prospectively.	Primary: Accuracy of the artificial intelligence mode Secondary: Failure rate of the artificial intelligence model (for example, due to poor quality or missing data)	Guy's and St Thomas' NHS Foundation Trust, UK

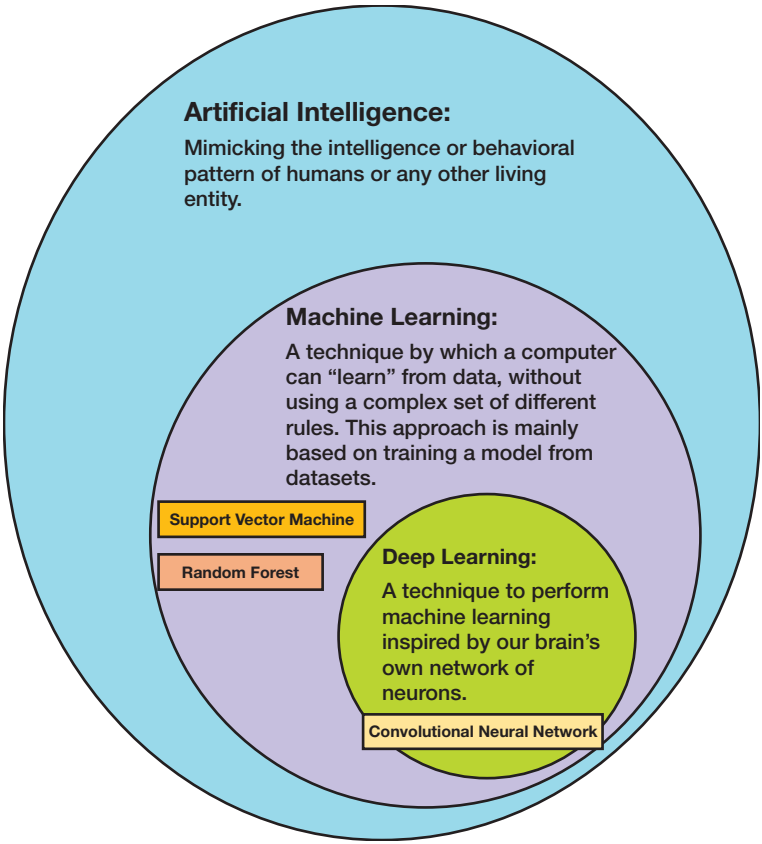

Figure 2. The relationship between Artificial intelligence (AI), Machine learning (ML) and Deep learning (DL) and models currently employed in survival analysis (26).

the outcome (27). ML and DL algorithms can be designed to capture the complexity of the patient profile by producing prognostic models that consider a large number of predictors including spatial and non-spatial data. Data extracted from large-scale registry and institutional data have been used for training ML and DL algorithms to improve the performance of survival outcome predictions. The data that have been used as training data include, but are not limited to, clinical characteristics, radiomics, histology, and molecular characteristics. Many studies have also shown different combinations of the use of imaging data (such as, Magnetic Resonance Imaging [MRI]) and Positron Emission Tomography [PET]) combined with clinical, histological, or genomic features (28–36). Some of the most popular ML algorithms used in survival prediction include support vector machine (SVM), random forest (RF) and a convolutional neural network (CNN) (37,38). Their relationship with respect to AI, machine learning, and deep learning is illustrated in Figure 2. All three methods require a training step where example data, called training data, is used as an input into a learning

algorithm which develops a model capable of mapping the input to an outcome as accurately as possible, and a prediction step where the trained model is used to predict outcomes given new data (37, 38). The individual approach inherent in each model is described below.

Support vector machine

SVM is a popular ML algorithm that has been used in classification and regression analysis in many fields of science. It can be successfully applied to analyze data with a large number of predictors and a limited sample size such as, thousands of radiomic features derived from imaging data, to predict survival outcome. The underlying principle of SVM is to optimize the separation of all observations into different classes (39). In the context of survival prediction, an SVM model can classify patients' survival time as long or short defined by an arbitrary survival time threshold used in model training. For example, Panesar *et al*. used the SVM algorithm to train models containing features including clinical and molecular characteristics, and WHO grade. A total of 76 patients were split into training and testing datasets in a 7:3 ratio randomly each time for 15 training cycles. The average performance of the resulting 15 models achieved a better binary classification performance (accuracy = 73.33%) for 2-year survival than the benchmark statistical regression methods (accuracy = 69.27%) (34). Similarly, Sanghani *et al*. extracted 2200 radiomics features, including texture, volume and shape, from multi-channel MRI data of 163 patients and combined them with clinical features such as age and KPS. These features were used to train an SVM model which selected the top 150 most predictive features and used them to produce a model that classifies each patient's overall survival (OS) time into 2 classes (greater or less than 400 days) and 3 classes (<10, 10–15, >15 months). The prediction accuracy was 98.7% for 2-class, and 88.95% for 3-class in cross-validation using internal data (29). Efforts have been made to adapt the SVM model for time-to-event analysis to predict survival time and improve its performance on right censored data by Khan *et al*. (40) and Van Belle *et al*. (41) by integrating regression constraints.

Random forest

Random forest (RF) is a non-parametric ML algorithm that constructs multiple decision trees based on training features and uses the consensus or average of their output to get a more accurate prediction. Similar to SVM, RF algorithm can be used to model a large number of predictors with a limited number of observations (18, 42). For survival analysis, RF has been adapted by Ishwaran *et al*. to create a Random Survival Forest (RSF) capable of time-to-event analysis, taking into account right censored data (42). Audureau *et al*. trained two random forest models with different approaches, using clinical features including demographics, tumor location, KPS and treatment from 407 patients as a training set. The RF models performed slightly better than the gold standard statistical regression model (CPH model) when validated using external validation data (370 patients) with C-index of 70.14 and 70.37. Both RF models also identified KPS as the most important feature for predicting OS (43). Chang el al. trained a random

forest model using features including volumetric, shape, texture, parametric, and histogram extracted from pretherapy (2293 features) and post-therapy (9811 features) multimodal MRI images of 84 patients to predict progression free survival (PFS) and OS. Long and short survival was defined as surviving more and less than the 50th percentile of PFS or OS in the training cohort respectively. An accuracy of 0.76 in classifying long and short survival was achieved in the validation cohort when using both pre- and post-therapy features, greatly exceeding the prediction accuracy of models trained only using pre-therapy features which was 0.57 and 0.54, respectively (35).

Convolutional neural network

Convolutional neural network (CNN) is a deep learning technique widely used in image analysis. A CNN can learn to classify or identify objects in images by automatically learning to extract features from them instead of using human defined features to interpret the images. For example, Mobadersany *et al*. developed a survival convolutional neural network (GSCNN) that integrated raw histological images with genomic biomarkers (IDH mutation status and 1q/19q codeletion) from 769 patients to produce a survival prediction framework with prognostic accuracy (C-index = 0.801) surpassing the WHO paradigm based on genomic classification or histological grading (36) when tested using internal data. Nie *et al*. trained a CNN to classify segments of whole brain images from 68 patients into long and short survival time (threshold at 650 days) with over 80% accuracy. The "deep features" learned by CNN, along with clinical features of patients, were then used as input for an SVM model which achieved an accuracy of over 90.66% in classifying long and short survival when validated on independent dataset (32). These studies highlighted the ability of CNN to automatically extract features predictive of survival from raw images. The generalizability and transferability of these high-level image features still require testing using data from various external institutions.

Evaluating machine learning approaches

Concordance index (C-index) is the most commonly used metric for evaluating survival predictions. It measures the accuracy of the model's predictions of the ordering of patients' risk of death (equivalent to survival time ranking) in comparison to ordering of patients' recorded survival time. It is a value ranging from 0.5 (indicating random ordering by model) to 1 (indicating perfect concordant ordering by model) and can also be estimated for right censored data (19, 44) (Table 1). Most ML and DL studies have achieved a C-index above 0.7 (31, 36, 45–47). For discrete survival classification using a survival time threshold, the overall classification accuracy is often calculated.

Radiomics and other computational approaches

Radiomics-type approaches are becoming increasingly common. They share an approach to inferring tumor grading, molecular features and/or tumor behavior following treatment in conjunction with tumor imaging and linking this to

outcome prediction including survival (46–48). Broadly, radiomics is an emerging field that involves extraction and quantification of features from medical images (48). The data can then be analyzed using computational analysis and models to identify predictive image biomarkers that characterize tumor behavior. Jan *et al*. carried out an extensive literature review of radiomics-based analyses, with a particular focus on computational modeling, machine learning, and fractal-based analysis aimed at optimizing differential diagnosis and predicting clinical outcomes. Han et al. combined a deep learning and radiomics model to predict OS in a cohort of 50 patients from their institution and 128 patients from TCGA with high-grade glioma. They calculated 348 radiomics features and 8192 deep features generated by a pretrained convolutional neural network and then applied feature selection and Elastic Net-Cox modeling to differentiate patients into long- and short-term survivors (46). Similarly, Lao *et al*. employed deep features to generate radiomic signatures for prediction of OS in a data set of 75 patients and an independent validation data set of 37 patients with glioblastoma. They extracted a total of 1403 handcrafted features and 98304 deep features from MRI and generated a radiomics nomogram combining the signature and clinical risk factors such as age and KPS. The proposed signature achieved better performance for prediction of survival and significant stratification of patients into prognostically distinct groups with a C-index of 0.739, demonstrating that a prognostic imaging signature exists and patient stratification for glioblastoma was possible (47). While most studies employ MRI, functional imaging in the form of ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG PET) has also been employed in radiomics analyses (30,49). Li *et al*. employed the images of 127 patients to develop a model to analyze features reflecting glioma metabolism for predicting IDH genotype and prognosis and generated a radiomic signature significantly associated with IDH genotype (49).

Imaging and tumor grading-based survival prediction

By far the most common theme in computational glioma survival prediction involving imaging relates to glioma tumor grading. Numerous publications have employed tumor grading from imaging features as a starting point to help develop prognostic biomarkers (21, 50–60). Some publications have focused strictly on glioma grading (31, 50, 58) while others explored more specific molecular subtypes (54) and IDH mutation status (51–53, 57, 58). Juan-Albarracin *et al*. developed ONCOhabitats (https://www.oncohabitats.upv.es [accessed on 10 December 2020]): an online open access system for glioblastoma analysis based on MRI data, including malignant tissue segmentation and vascular heterogeneity assessment of the tumor while implementing a deep patch-wise 3D CNN with residual connections. This allows open-access services for glioblastoma heterogeneity assessment and medical image analysis with a computational capacity of 300 cases per day (50). Tan *et al*. analyzed the clinical data, genetic features and MRI images of 147 high-grade glioma (112 patients as training cohort, 35 as independent test cohort) to develop a radiomic signature to predict OS for HGG, and constructed a nomogram by combining selected radiomic, genetic and clinical risk factors. The radiomics features were extracted from the tumor area and the peritumoral edema area on CE-T1WI (contrast-enhanced T1-weighted imaging)

and T2FLAIR (T2 fluid-attenuated inversion recovery) images, and the risk factors and OS were explored by Kaplan-Meier survival analysis by stratifying patients into low- and high-risk groups (C-Index 0.707 and 0.711 in training and test cohorts respectively) (31). By contrast, using CNN Zhuge *et al*. created two fully automated glioma grading methods on conventional MRI images that were then evaluated on The Cancer Imaging Archive (TCIA) LGG data, and the BraTS Benchmark 2018 training datasets (55). In yet another approach, Liao *et al*. examined multi-dimensional MRI features extracted from segmented lesions of T2-FLAIR MRI data of 137 glioblastoma patients together with RNA sequencing in groups of glioblastoma patients identifying radiomic parameters including intensity, shape and textural features that were incorporated into seven classes to divide the patient cohort into two groups depending on their survival time, concluding that MRI features are predictive of survival outcomes and image features are highly associated with selective metagenes (59).

With respect to molecular classification in terms of IDH mutation status, Suchorska *et al*. stratified 301 patients with WHO grade II (n = 181) or grade III glioma (n = 120) according to their molecular profile and reviewed pre-operative MRI and volumetric analyses of contrast-enhanced and T2 volumes, showing that contrast enhancement on initial MRI is a prognostic factor for survival with dependence on volume distinguishing IDH-mutated from IDH-wild type tumors (51). Similarly, with an eye towards predicting IDH mutation pre-operatively, several publications have employed MRI and ML (57, 58, 61). Zhang *et al*. employed pre-operative MRIs of 120 HGG patients with confirmed IDH genotype, to extract 2970 imaging features from pre- and post-contrast T1-weighted, T2-weighted, and apparent diffusion coefficient (ADC) maps. Using RF, the preoperative MRI features with the highest predictive value for IDH genotype were patient age, parametric intensity, texture, and shape (57). Similar analyses using preoperative prediction of IDH status were carried out by Tan *et al*. in 105 astrocytoma (Grades II-IV) (58).

Imaging and response to treatment

The use of imaging to assess response to treatment in glioma is of tremendous importance for both patient management and outcome assessment. With respect to computational approaches on analyzing survival outcomes, research to date has focused on two main aspects: (i) distinguishing progression from pseudo-progression (62, 63); and (ii) connecting systemic therapy or radiation therapy (RT) to imaging changes (64). Artzi *et al*. utilized data generated using conventional, dynamic contrast enhanced (DCE)-MRI and magnetic resonance spectroscopy from 20 patients to extract the enhanced lesion area using independent component analysis and choline/creatine values and compared treatment-related changes with normal-appearing white matter. They identified a progressive disease component within the lesion, concluding that the results may have clinical importance for pre-operative planning, guidance for targeting biopsy and early prediction of radiological outcomes in patients with HGG (62). Kebir *et al*. carried out a similar analysis using 14 patients with HGG suspected of progression but used FET-PET imaging to identify 3 clusters based on 10 predominantly textural FET-PET features. Similar studies were also carried out by Chan *et al*. and Petrova *et al*. (64, 65).

Correlating response to RT with images using ML and DL is challenging. Mizutani *et al*. employed scans of 35 patients with malignant glioma, identifying 12 clinical features and 192 dose–volume histogram (DVH) features and used SVM to predict OS times after RT. They found that prediction accuracy was significantly improved with the combined use of clinical and DVH features compared with the separate use of each feature (66). Qiu *et al*. compared RSF and traditional CPH to predict tumor progression after particle beam radiotherapy in 82 HGG patients and found that CPH demonstrated a better performance in terms of integrated C-index as compared to the RSF model (18).

The question as to the most optimal computational approach to be employed in glioma survival prediction pertaining to imaging data is as yet unresolved (32, 55, 67–69). Zacharaki *et al*. (28) suggested that prediction models based on data-mining algorithms can provide a more accurate information about prognosis of malignant gliomas than histopathologic classification alone. Since then, more studies have concluded that the combination of clinical and multi feature imaging is crucial to obtain accurate model prediction (39, 45, 70). Some studies also suggest that DL approaches may be superior to ML (32, 67, 68). Mirroring traditional RPA approaches to survival prediction, some publications aim to classify gliomas by survival time using DL techniques (71) and more novel approaches. For example, Smedley *et al*. describe a neural network-based approach that takes high-dimensional gene expression data as input and performs non-linear mapping to an imaging trait, identifying imaging traits with specific transcription patterns, such as edema and genes related to cellular invasion (72). Mostly, small institutional cohorts are being employed as opposed to large registries such as TCGA (73, 74). Studies suggests that imaging-based glioma survival prediction carries greater potential as compared to traditional approaches to what is currently radiographically being identified as gross tumor on scans (67, 74). Brain tumor segmentation also remains an active area of research that has significant implications for computational approaches (75–77). Currently, most studies are based on very small institutional cohorts (Figure 1) and the validation of models is heterogeneous in the literature, although with ongoing research this is likely to change rapidly in the coming years.

Molecular and genetic characterization of glioma, digital pathology, and survival prediction

Several molecular markers such as IDH1/2, 1p19q co-deletion, TERT and MGMT promoter methylation, G-CIMP methylation, EGFR alternations, BRAF V600E mutations and histone mutations have been found to have prognostic significance in glioma (78). Statistical methods can be applied to stratify prognosis based on molecular characteristics. Bell *et al*. created a new RPA model (NRG-GBM-RPA) that creates distinct prognostic groups based on age, MGMT protein and c-Met protein levels. The new model resulted in improved survival stratification in patients with glioblastoma treated with RT and temozolomide in comparison with current RPA classifications based on age, KPS, resection status and neurofunction status (79).

In the 2019 “Contributions from the 2018 Literature on Bioinformatics and Translational Informatics” (80), the two primary trends identified were:

(i) the adoption of artificial intelligence and DL methods in medical informatics; and (ii) the implementation of a pan-cancer approach and integration of multi-omics data for more insightful analyses (80). In glioma, RNA based biomarkers are evolving and traditional statistical models have been employed to create prognostic groups (81, 82), while ongoing efforts using computational approaches that involve ML and DL are advancing for somatic copy number variations (83) and gene expression microarrays (84). To date, both 1p19q (85) and MGMT (86, 87) are important molecular features undergoing active inclusion into computational approaches to glioma survival prediction. Transcriptomic analyses using TCGA glioma expression datasets are being advanced to identify novel tumor subcategories using ML (88, 89). Panesar *et al*. applied 3 ML techniques (CNN, RF, SVM), and classical logistic regression to the molecular characteristics of a dataset consisting of 76 patients with glioma of all grades achieving reasonable performance compared with similar studies in the literature, but noted that, similar to other studies, traditional statistical methods were of similar benefit (34). This illustrates that more research is needed particularly with larger data sets and validation.

Digital pathology is emerging as an increasingly important facet of the approach to glioma pathology and classification and has been employed in both ML and DL approaches to integrate information from both histology images and genomic biomarkers to predict time-to-event outcomes (36). It has been utilized for whole-slide imaging of histologic sections to extract quantitative features (90). Powell *et al*. used hematoxylin- and eosin-stained slides from TCGA to create a machine learned dictionary of "image-derived visual words" associated with survival outcomes while connecting image-derived phenotypic characteristics with molecular signaling activity and the behavior of low-grade glioma (91).

CONCLUSION

Retrospective spatial and nonspatial data from patients with glioma is increasingly available and prospective data is being generated to provide an avenue for novel approaches to survival prediction. While many computational approaches show promising performance in terms of survival prediction accuracy, most ML prognostic models are trained using data from single-institutions and have not been validated using external cohorts. To facilitate the implementation of ML prognostic models into clinical practice, prospective validation of these models on large scale heterogenous cohorts from multiple centers would be required (92). Digital pathology is an exciting avenue being advanced to explore survival prediction in glioma. Most importantly, the neuro-oncology field needs to familiarize itself with computational approaches and quality metrics for the assessment of such approaches to ensure robust conclusions that drive improvement in patient outcomes in the clinic. There is a growing need to foster reliable clinician/ML innovation to support the generation of robust data sets in large scale registries such as TCGA, SEER and BraTS. Efforts towards developing consensus and clinical oversight in the methods for data acquisition and coding across different institutions could facilitate external and prospective validation of survival prediction models.

Conflict of Interest: The authors declare no potential conflict of interest with respect to research, authorship and/or publication of this chapter.

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Molecular Markers of Gliomas to Predict Treatment and Prognosis: Current State and Future Directions

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Abstract: Gliomas used to be classified mainly based on histopathological criteria. In 2016, the Word Health Organization introduced a new classification system incorporating the molecular profile of gliomas. This has prompted research on the utility of molecular signature of gliomas to predict prognosis and response to therapy. While experimental data appear to be promising, the clinical use of molecular markers to predict prognosis and drive individual treatments is still a challenge. This chapter presents an overview of the major genes and markers associated with the characterization and development of gliomas, and the potential of these molecular markers in clinical decision-making. The current challenges and future directions are discussed.

Keywords: brain cancer; clinical trials; glioblastoma; molecular markers; therapy

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INTRODUCTION

Malignant gliomas can develop anywhere in the central nervous system (CNS), but they mainly occur in the brain, accounting for about 80% of all primary malignant brain tumors in adults (1). Based on histology, gliomas are traditionally divided into diffuse and non-diffuse gliomas. Diffuse gliomas are characterized by intense infiltrative growth into the surrounding parenchyma of the CNS whereas non-diffuse gliomas are more circumscribed (2, 3). Based on the degree of anaplasia, gliomas are graded into non-infiltrating astrocytomas (grade I), diffuse astrocytomas (grade II), anaplastic astrocytomas (grade III) and glioblastoma (grade IV), the most aggressive form (4). Histological diagnosis is based on atypical cell morphology, variation in nuclear size and shape, cell density, mitotic activity, necrosis and vascular properties. Although this classification system based on histology has evolved over the years, there are limitations in diagnostic accuracy, such as being subject to significant interobserver variability, with occasional disagreements between neuropathologists (5). Advances in understanding of molecular pathology of gliomas have led to the incorporation of molecular subtypes of gliomas in the 2016 World Health Organization (WHO) CNS tumor classification (6, 7). Current knowledge on the molecular signature of gliomas, how this classification is already driving decisions on treatments and predicting prognosis, and the challenges for using this information in clinical practice are discussed in this chapter.

MOLECULAR CHARACTERIZATION OF GLIOMAS

During the development of gliomas, genetic and epigenetic changes can culminate in the loss of function of tumor suppressor genes, for example, decreased expression of tumor suppressor protein 53 (TP53), phosphatase and tensin homolog (PTEN), cyclin-dependent kinase inhibitor 2A (CDKN2A) and retinoblastoma protein (RB); or overexpression of oncogenes, such as rat sarcoma (*RAS*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha *(PIK3CA),* cyclin dependent kinase 4 *(CDK4),* murine double minute 2 *(MDM2)* and epidermal growth factor receptor *(EGFR)*. The loss of TP53 and the consequent activation of the RAS pathway, through inactivation of the neurofibromatosis type 1 *(NF1),* would be sufficient for the development of diffuse malignant astrocytoma (8, 9). This specific change, together with histopathological knowledge, can enable a better diagnostic accuracy and precise prediction of prognosis and responsiveness to antitumor treatments (10).

Diffuse gliomas

In the WHO 2016 classification, diffuse gliomas (whether astrocytic or oligodendroglial) are grouped not only by behavioral, histological, or growth patterns, but also by punctual genetic changes in the isocitrate dehydrogenase 1 or 2 (*IDH1* and *IDH2*) (3, 11, 12). Mutations in the 132 or 172 codons of the *IDH1* and *IDH2* genes, respectively, are present in 80–90% of low-grade gliomas (WHO grade II/III), and in approximately 5% of glioblastomas. These mutations result in

neo-enzymatic activity that culminates in the production of 2-hydroxyglutarate. This causes high histone methylation and hypermethylation of multiple CpG islands; the *IDH* mutations are possibly one of the first genetic changes that occur during glioma tumorigenesis (10, 12, 13). However, this alone would not be enough for the development of these types of tumors. *IDH*-mutant astrocytomas, for example, show additional mutations in the *TP53* and alpha thalassemia/mental retardation syndrome x-linked (*ATRX*) genes, the latter leading to a loss of the nuclear expression of the transcriptional regulator *ATRX*, which is important for the remodeling of chromatin and regulation of telomere length (1, 10). It soon became clear that tumors with the same histological classification, but with a different *IDH* grading, (*IDH*-mutant or *IDH*-wildtype), had different clinical outcomes. In addition, several *IDH*-wildtype gliomas in adults, classified histologically as diffuse grade II and III, display molecular characteristics and behavior similar to glioblastoma (grade IV) (11, 14, 15). These observations led to the recognition of the *IDH* mutation as a suitable biomarker for the categorization of gliomas, introducing the following genetically defined subtypes: anaplastic astrocytoma *IDH*-mutant, diffuse astrocytoma *IDH*-mutant, glioblastoma *IDH*-mutant and oligodendroglioma *IDH*-mutant. In addition, categories for diffuse *IDH*-wildtype gliomas have also been created, constituting additional classifications. It is important to highlight that the 2016 WHO classification includes a category entitled not otherwise specified (NOS), for cases in which molecular tests were inconclusive or could not be performed (11, 16). The determination of the *IDH* mutation status is, therefore, essential for the integrated classification of the glioma, which is made possible by immunohistochemistry, using mutant specific antibodies–*IDH1R132H* being the most common (7). This new characterization of tumors based on the molecular concept, however, is still in its early stages, and more studies that contribute to the identification of the molecular profile of the tumor are needed.

In addition to the presence of the *IDH* mutation, oligodendroglial tumors demonstrate allelic loss of chromosome 1p and 19q (1p/19q codelection), which is associated with a favorable prognosis in relation to tumors without this codelection. Many astrocytomas are *IDH*-mutant and do not have the 1p/19q codelection (7, 13). In this way, the *IDH*-mutant gliomas can include two main groups: (i) oligodendrogliomas, which, in addition to the *IDH* mutation, have the 1p/19q codelection and activating mutation of the telomerase reverse transcriptase (*TERT*) promoter (mutations in this region increase the expression of *TERT*, essential for the proliferative character of cancer); and (ii) astrocytomas, characterized by the presence of the *IDH* mutation and frequent mutation in *ATRX* and *TP53* (7, 11, 17). The low-grade gliomas without the *IDH* mutation are called *IDH*-wildtype and are considered a provisional entity by 2016 WHO classification (7, 15). Most low-grade gliomas have *IDH* mutations, and 1p/19q codelection is frequent in oligodendrogliomas; these are prevalent in young adults and their prognosis is favorable, including better responses to radiotherapy and longer survival, compared to diffuse gliomas without these mutations (9, 13).

Glioblastomas

Glioblastomas have also been reclassified into distinct subtypes. About 90% are now classified as *IDH*-wildtype; they are located in the supratentorial brain region

and are characterized by extensive necrosis, *TP53* mutations, *TERT* promoter methylation, *EGFR* amplification and *PTEN* mutations. Approximately 10% of cases are *IDH*-mutant glioblastoma, considered secondary, which means they may have progressed from a lower grade glioma; they are located in the cerebral frontal lobe and are characterized by limited tumor necrosis, *TP53 and ATRX* mutations, and *TERT* promoter mutation (18–20). Overexpression of platelet-derived growth factor receptor alpha (*PDGFRA*) gene and *IDH1* mutation are among the main genetic changes found in low-grade gliomas, as well as in secondary glioblastomas (21). Therefore, based on molecular studies, four glioblastoma subtypes are currently classified: (i) classic (high level of *EGFR* amplification), (ii) mesenchymal (*NF1* mutation or loss), (iii) neural (*EGFR* overexpression) and (iv) proneural (amplification of *PDGFRA*) (22).

Another example is the methylation of the O6-methylguanine-DNA methyltransferase (*MGMT)* promoter (3). The methylation of the *MGMT* promoter in CpG regions of the DNA correlates with a favorable response to alkylating agents and results in an epigenetic silencing (decreased expression) of the *MGMT* protein, reducing the repair activity by this protein. This methylation is observed in approximately 40% of all glioblastomas. Higher promoter methylation levels predict longer survival for patients with glioblastoma (22 months, versus 13 for patients with unmethylated tumors) (3, 23).

MOLECULAR PROFILE GUIDING CLINICAL PRACTICE

According to the European Organization for Research and Treatment of Cancer (EORTC) 22844 study, the factors that confer poor prognosis for low-grade gliomas are: (i) age equal to or older than 40 years; (ii) histology compatible with astrocytoma; (iii) tumor diameter equal to or greater than 6 cm; (iv) tumor that exceeds the midline; and (v) presence of neurological deficits prior to surgery (24). In this context, patients who have any of the two of the factors are considered low-risk, and the mean overall survival is 7.8 years; those who have three or more factors are considered high-risk and the mean overall survival is 3.7 years (25). The presence of *MGMT* methylation, *IDH* mutation, or both, associated with 1p/19q codelection offer a better prognosis with a greater overall survival. However, *MGMT* methylation, *IDH* mutation, and *TP53* immunopositivity were associated with a higher rate of malignant transformation of tumors (26).

The extent of surgical resection is a predictor of overall survival, epileptic seizures and tumor recurrence (27, 28). Complete resection of the lesion provides a better prognosis. Some studies suggest that a supratotal resection reduces the incidence of malignant transformation, decreases tumor recurrence, and promotes greater survival in low-grade gliomas (29, 30). The correlation between the molecular type and the degree of resection suggests differences in prognosis between astrocytomas (mean survival = 10.9 years) and oligodendroglioma (mean survival = 17.1 years). In grade II astrocytomas (*IDH*-mutant), a tumor residue with a volume of 0.1 to 5.0 cm^3 is associated with a significant reduction in survival compared to total tumor resection whereas in grade II oligodendrogliomas (*IDH*-mutant, 1p/19q codeletion), there is no such difference. This is also the case with grade III anaplasic astrocytomas (*IDH*-mutant) and anaplastic

oligodendrogliomas (*IDH*-mutant and 1p/19q codeletion) (31). This reinforces the importance of surgical radicality, especially in the case of tumors of astrocytic lineage, which by nature have a worse prognosis than tumors of oligodendroglial lineage. In addition, these studies demonstrate the clinical relevance of the molecular signature of the tumor to predict the prognosis after surgical resection. Despite these advances, the use of this knowledge to assist in clinical management and the development of effective therapies has not yet been consolidated and systematized. The standard treatment, in most countries, still consists of maximum safe resection associated with radiotherapy and chemotherapy (Figure 1) (31, 32).

For glioblastoma, the factors for good prognosis are age less than 45 years, Karnofsky Performance Scale (KPS) greater than 80%, and degree of resection greater than 78% (33–35). Liang *et al.* (32) showed that low KPS (<85%) is an independent risk factor for death in the first year of treatment of patients, regardless of histological grade. The degree of surgical resection and adjuvant radio and chemotherapy (temozolomide - TMZ) is associated with increased overall survival and delayed tumor progression, despite the high recurrence of gliomas (36). At molecular level, *MGMT* promoter methylation, 1p/19q codeletion and *IDH1* mutation are markers and predictors of a favorable prognosis. Tumor-treating fields in combination with TMZ increase overall survival and disease-free survival compared to TMZ alone (37). However, tumor-treating fields is not a target-driven

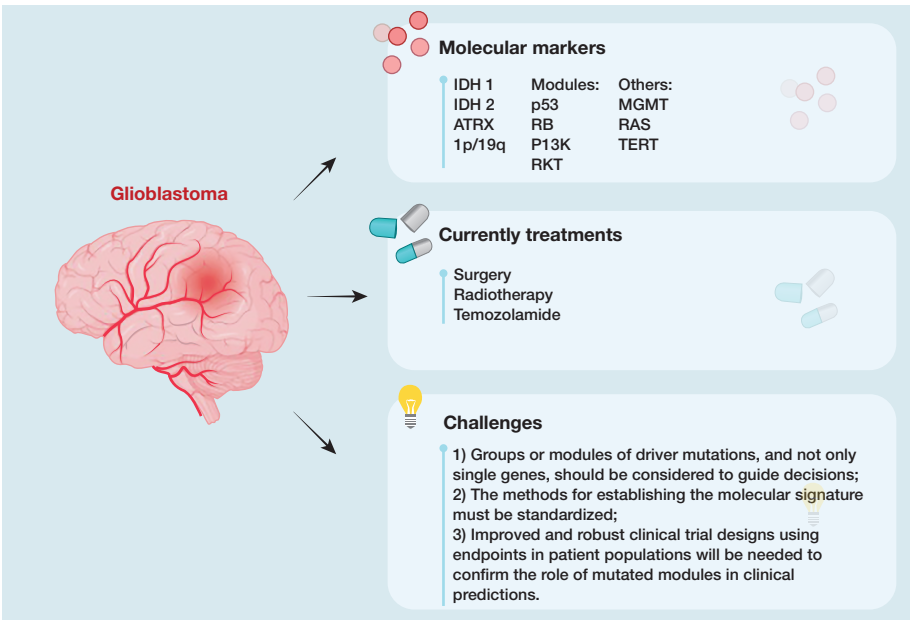

Figure 1. Molecular markers and current challenges. The schematic representation includes the markers and modules listed in the chapter, as well as the main therapies for the treatment of glioma and the challenges to use tumor molecular signature to guide treatments and predict prognosis.

therapy and there is no study correlating the clinical response with the molecular profile of the tumor.

In the EORTC phase III study, 437 patients with recurrent glioblastoma after radio and TMZ therapy were randomized to receive lomustine or lomustine plus bevacizumab (38). *IDH* mutations and *MGMT* promoter methylation were monitored. The combination therapy demonstrated a marginal increase in progression-free survival (1.5 months for lomustine and 4.1 months for the combination) without any improvement in overall survival. The BRAF V600E gene mutation has been identified in approximately 50% of glioblastomas (39). In a phase II study of 24 patients with the mutation, vemurafenib, an inhibitor of the BRAF kinase domain, demonstrated a lasting response and stable disease for 12.9 months (40).

While the limited prognostic value of *IDH1* mutations have been demonstrated, little is known about other genes often mutated in gliomas, including *TP53, PTEN, CDKN2A, RB1, EGFR, NF1, PIK3CA,* phosphoinositide-3-kinase regulatory subunit 1 *(PIK3R1)* and others. Although many clinical trials are underway (Table 1), to the best of our knowledge, no study has been published so far in which the treatment decisions were made based on the molecular classification of the glioma. *MGMT* methylation status remains the most reliable tumor biomarker, as it can be used to predict the tumor's response to the therapy with TMZ (41). Also, the 1p/19q co-deletion is a consistent marker, and has been used as a molecular signature of oligodendroglial tumors, predicting the response to vincristine chemotherapy of anaplastic gliomas (42). However, before adapting the molecular classification in clinical practice, further studies are needed.

FUTURE DIRECTIONS

Based on the current knowledge, it is possible to define three main challenges that need to be considered going forward to be successful in predicting prognosis and treatment: (i) identifying functional modules or groups of driver mutations, rather single genes; (ii) standardization of methods for establishing molecular signature; and (iii) molecular markers-based clinical trial designs.

Functional modules

The first challenge is identifying functional modules, a set of altered genes with functional relevance in gliomas. Cerami *et al.* (43) observed that genetic alterations in glioblastoma tend to occur within three main specific functional modules: *p53*, *RB* and *PI3K*. Each module contains groups of mutations. For example, the gene alterations identified in module *p53* are *TP53*, *MDM2*, and mouse double minute 4 - *MDM4;* the *RB* module are *RB1*, *CDK4*, and *CDKN2A;* and the *PI3K* module are *PIK3CA, PIK3R1, PTEN*, and insulin receptor substrate 1 (*IRS1*). In addition to these three modules, the receptor tyrosine kinases (*RTK*) module has been described. It contains amplification or mutation of the *EGFR* gene, *BRAF* mutation, neurotrophic tyrosine receptor kinase (*NTRK*) or fibroblast growth factor receptor (*FGFR*) gene fusions, and amplification or fusion of the *MET* gene (44).

TABLE 1 Clinical trials correlating treatment and molecular profile of gliomas

Name	Description	Observations
Molecular profiling in guiding individualized treatment plan in adults with recurrent/progressive glioblastoma (TGEN)	Tumor tissue from patients with glioblastoma undergoing surgery is collected for analysis of the molecular profile together with DNA from blood samples. Drugs could be suggested, and the molecular data will be correlated with tumor progression and patient survival in treated and untreated groups.	**Status:** Completed **Country:** USA **Last update:** posted: Jul 28, 2020 **ClinicalTrials.gov identifier:** NCT02060890
Chemotherapy and radiation therapy for the treatment of IDH wildtype gliomas or non-histological molecular) glioblastomas	Phase II study that evaluates the relationship between the use of temozolomide and radiotherapy in the treatment of patients with low-grade gliomas, IDH-wild type glioblastoma or non-histological molecular glioblastomas.	**Status:** Recruiting **Country:** USA **Last update:** posted: Nov 10, **2020** **ClinicalTrials.gov identifier:** NCT04623931
Feasibility of individualized therapy for recurrent GBM	A pilot study that evaluates the feasibility of implementation for individualized treatment based on a report of the genetic profile of patients with recurrent surgical glioblastoma.	**Status:** Active, not recruiting **Country:** USA **Last update:** posted: Apr 28, **2020** **ClinicalTrials.gov identifier:** NCT03681028
Clinical benefit of using molecular profiling to determine an individualized treatment plan for patients with high grade glioma (PNOC008)	The study evaluates a new treatment approach based on patient's tumor gene expression in children with high-grade gliomas.	**Status:** Recruiting **Country:** USA **Last update:** posted: Dec 04, **2020** **ClinicalTrials.gov identifier:** NCT03739372
Treatment response and prognosis in glioma patients: Q Cell and its biological characteristics	The study analyzes the molecular markers of patients with glioblastoma that will be evaluated qualitatively and quantitatively, comparing their relationship with survival/survival free of progression and response to treatment.	**Status:** Unknown **Country:** China **Last update:** posted: Jan 28, **2014** **ClinicalTrials.gov identifier:** NCT02047058

Table continued on following page

TABLE 1 **Clinical trials correlating treatment and molecular profile of gliomas (Continued)**

Name	Description	Observations
Pilot study of Abemaciclib with Bevacizumab in recurrent glioblastoma patients with loss of CDKN2A/B or gain or amplification of CDK/6	The study describes the effects of Abemaciclib administered with Bevacizumab in patients with recurrent glioblastoma with specific molecular changes.	**Status:** Recruiting **Country:** USA **Last update:** posted: Jan 07, **2020** **ClinicalTrials.gov identifier:** NCT04074785
Imetelstat sodium in treating younger patients with recurrent or refractory brain tumors	This phase II molecular biology study looks at how sodium imetelstat works in treating younger patients with refractory or recurrent brain tumors.	**Status:** Terminated **Country:** USA **Last update:** posted: Jul 20, **2018** **ClinicalTrials.gov identifier:** NCT01836549
NCT Neuro Master Match - N^2M^2 (NOA-20) (N^2M^2)	The study assesses the improvement of overall survival of patients with glioblastoma with an unmethylated MGMT promoter based on molecular characterization and use of targeted compounds in a trial design.	**Status:** Recruiting **Country:** Germany **Last update:** posted: Feb 27, **2020** **ClinicalTrials.gov identifier:** NCT03158389
Lapatinib in treating young patients with recurrent or refractory central nervous system tumors	This phase I/II trial studies lapatinib to see how well it works in treating young patients with recurrent or refractory central nervous system tumors also correlating with molecular aspects.	**Status:** Completed **Country:** USA **Last update:** posted: May 23, **2014** **ClinicalTrials.gov identifier:** NCT00095940
Improving the selection of patients with glioblastoma multiforme for treatment with epidermal growth factor receptor inhibitor therapies	The study assesses biomarkers that can improve patient selection for therapies with epidermal growth factor receptor inhibitors.	**Status:** Completed **Country:** USA **Last update:** posted: Jan 24, **2018** **ClinicalTrials.gov identifier:** NCT00897663

Zhang *et al.* (45) searched for mutated core modules in glioblastoma and ovarian carcinoma datasets and identified five and two mutated modules, respectively. For glioblastoma, cyclin-dependent kinase inhibitor 2B (*CDKN2B)* and *CDK4* for the *RB* module, and *EGFR* and *NF1* for the RTK module were detected.

The International Cancer Genome and The Atlas of the Cancer Genome (TCGA; in which glioblastoma was the first tumor examined) are initiatives to understand the genetics of tumors, helping in the generation of new therapies

and better diagnostic methods. With these platforms, researchers from several countries use samples of gliomas from multiple centers to carry out comprehensive molecular characterization (9, 17, 46). Using the TGGA database, Gu *et al.* (47) identified multiple co-occurring alterations among the three modules mentioned above (TP53, RB and RTK). For example, simultaneous co-alterations in *RTK* and *TP53* modules were present in 31 glioblastoma patients. Forty-one glioblastoma samples carried alterations in the *RTK*-related module, which consisted of *EGFR*, *PIK3CA*, phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta (*PIK3C2B*), ATP synthase F1 subunit beta (*ATP5B*) and mitogen-activated protein kinase 14 (*MAPK14*). Thirty-five samples were detected with alterations in the p53-related module, including *CDKN2A*, *MDM4*, E1A binding protein p300 (*EP300*), *CD4*, major histocompatibility complex class II (MHC II), DR alpha (*HLA-DRA*), diacylglycerol kinase gamma (*DGKG*) and heat shock protein 90 alpha family class A member 1 (*HSP90AA1*). Alterations within the *RB* module included amplifications of cyclin-dependent kinase inhibitor 1B (*CDKN1B*) and mutations of *RB1*, IQ motif containing GTPase activating protein 1 (*IQGAP1*), WEE1 G2 checkpoint kinase (*WEE1*) and ephrin type-A receptor 7 (*EPHA7B*).

Taken together, it is possible to establish four main modules for the molecular signature of gliomas: (i) *p53* (including *CDKN2A/2B, TP53, MDM2, MDM4, EP300, CD4, HLA-DRA, DGKG* and *HSP90AA*); (ii) *RB* (including *CDKN1B, CDKN2B, RB1, IQGAP1, WEE1, EPHA7By*, *CDK4* and *CDKN2A*); (iii) *PI3K* (including *PIK3CA, PIK3R1, PTEN*), and (iv) *IRS1* and *RTK* (including *EGFR, PIK3CA, PIK3C2B, ATP5B, MAPK14, BRAF, MET, NTRK, FGFR* and *NF1*). Other genes involved in each module, as well as new modules, can be further identified, since this field is still emerging.

Tang *et al.* (42) identified a module with four genes associated with survival: c-type lectin domain family 5 member A (*CLEC5A*), fibromodulin (*FMOD*), FKBP prolyl isomerase 9 (*FKBP9*) and galectin 8 (LGALS8) (48). CLEC5A/MDL-1 is a member of the myeloid C-type lectin family expressed in macrophages and neutrophils; *FMOD*, a glioblastoma-upregulated gene, promotes glioma cell migration through its ability to generate the formation of filamentous actin stress fibers; *FKBP9* is a peptidyl–prolyl isomerase and it has been implicated in neurodegeneration, mainly through accelerating fibrillization; and *LGALS8* plays functional roles in promoting glioblastoma cell proliferation and clonal sphere formation.

Based on RNA-Seq from TGGA database, Xu *et al.* identified a module with four genes related to prognosis: oncostatin m receptor (*OSMR*), SRY-box transcription factor 21 (*SOX21*), mediator complex subunit 10 (*MED10*) and protein tyrosine phosphatase receptor type N (*PTPRN*) (49). *OSMR* encodes a member of the type I cytokine receptor family; *SOX21* functions as a tumor suppressor during glioma genesis; *MED10* is a component of the coactivator for DNA-binding factors that activate transcription via RNA polymerase II; and *PTPRN* is a member of the protein tyrosine phosphatase family and may be involved in cancer initiation and progression. Although the specific mechanisms of glioma progression remain to be fully elucidated, these modules can be of assistance in studying the progression and prognosis of gliomas and help develop novel therapeutics and guide clinical practice.

Standardization of methods

The second challenge is to have standardized methods for determining the molecular signature of tumors. When choosing techniques, it is important to consider complexity, reproducibility, and costs. In addition, sample collections must be standardized. To evaluate the main aberrations, such as 1p/19q co-deletion, *IDH* and histone H3 mutations, direct sequencing, multiplex ligation-dependent probe amplification and/or fluorescence *in situ* hybridization (FISH) are used. However, these methods are complex and costly. Real-time quantitative PCR (qPCR) and immunohistochemistry have also been used and are considered simple and accurate techniques in the daily diagnosis, readily available for a small scientific facility (50). However, new diagnostic resources that are simpler, faster and easier to standardize, with established sensitivity, specificity and predictive values, are necessary for molecular information in the clinic.

Research groups around the world are trying to improve methodology and establish protocols. Shi *et al*. collected tumor tissues using image-guidance by magnetic resonance (MR) from 26 cases of glioblastoma, showing that this approach increased the content and purity of the samples compared with manual extraction (51). An ongoing study (ClinicalTrials.gov Identifier: NCT04539431) is validating a new platform for molecular characterization of patients affected by glioma. This includes a series of faster and less expensive qPCR methodologies compared to the standard analyzes (DS, MS-PCR).

Another important point to consider is tumor samples bias, which can occur due to somatic events in the primary tumor, between the primary and metastatic sites, and among metastatic sites, generating intra-tumor molecular heterogeneity (52). Taking samples from different parts of the tumors and metastases can help minimize this problem. In addition, methods for biobanking of gliomas derived from patients can also be a very rich tool for future analysis. Jacob *et al.* developed methods for generating glioblastoma organoids with high reliability, exhibiting rapid and aggressive infiltration when transplanted into adult rodent brains (53). They demonstrated the usefulness of glioblastoma organoids for testing personalized therapies, correlating mutational glioblastoma profiles with responses to specific drugs. The organoids maintained many key characteristics of original glioblastoma. In their work, 96.4% of *IDH1*-wildtype tumors resulted in organoids, but *IDH1*-mutant and recurrent tumors showed reduced success rates.

Our research group is conducting a translational study at the "Hospital Central da Irmandade da Santa Casa de Misericordia de São Paulo", São Paulo, Brazil, characterizing the molecular signature of gliomas. The objectives are to improve diagnostic methods, prognostic predictions using simple and low-cost techniques, and correlate molecular profile with response to standard and new treatments. The study is approved by the Brazilian Research Ethics Committee (Comitê de Ética em Pesquisa – CEP, #15215219.5.0000.5404). Briefly, tumor samples from patients are processed for molecular analysis. The biomarkers evaluated include those described in the WHO 2016 glioma classification, as well as other related markers (including markers from the major known modules), as detailed in Figure 2. The establishment of each tumor lineage in culture, as well as tissue samples, will be used later for the creation of

a biobank. The results of the analysis of the molecular profile will be made available to the medical team and will be of great importance for directing the most appropriate and specific adjuvant treatment. The established lineages of tumors can be used later to confirm tumor profile and response to other treatments. Epidemiological statistics will also be generated to contribute to the mapping of cases of brain cancer in the Brazil Unified Health System (Sistema Único de Saúde - SUS).

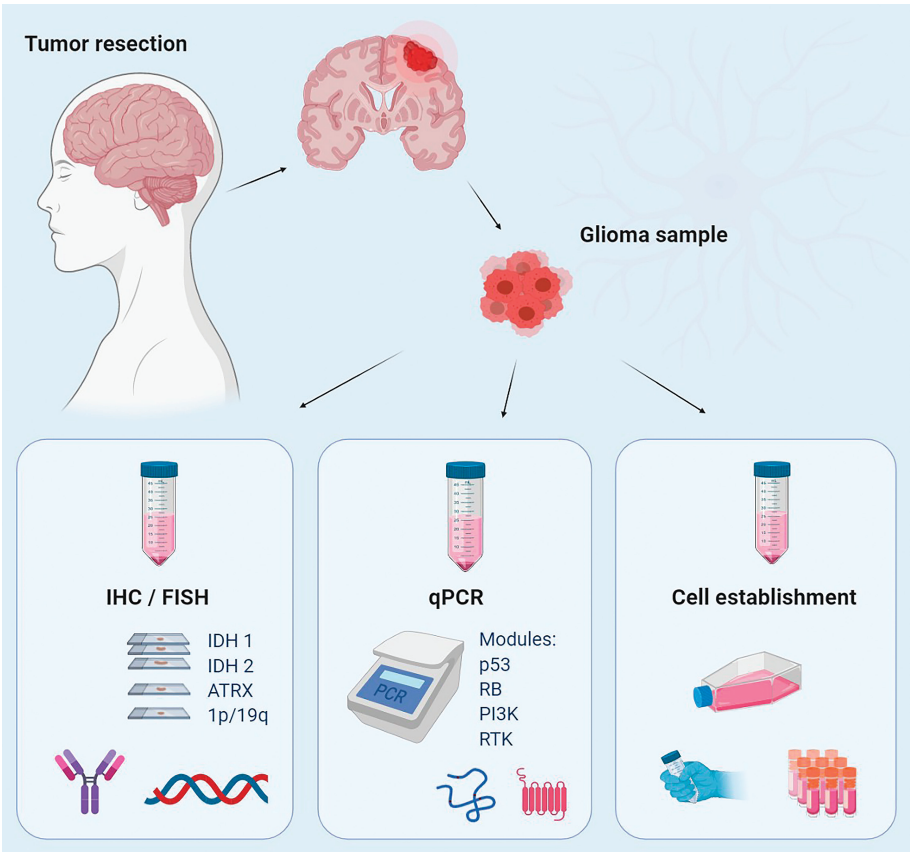

Figure 2. Schematic representation of the protocol for collecting and analyzing the molecular profile of glioma samples. After collection and adequate conditioning of the tumor sample, IHC and FISH techniques will be performed to determine the expression of the markers: IDH 1 and 2; ATRX and 1p/19q codeletion, respectively. qPCR analyzes will be carried out to evaluate the molecular markers modules: p53 (TP53), RB (CDKN2A), PI3K (PIK3CA), and RTK (EGFR and NF1). Other related molecular markers also will be evaluated such as MGMT and PDGFRA, AKT, mTOR, Rhoa, and ROCK (related to tumor development and also with cell migration and proliferation pathways, respectively). The establishment of the culture sample will analyze other parameters and also will be used, together with tissue samples, for the creation of a biobank.

Clinical trials

The third challenge is designing clinical trials based on the molecular signature of tumors. Although several studies are testing new drugs or combination therapies, only a few clinical trials are focused on establishing the effectiveness of individual treatments driven by the molecular profile of the tumor. Some studies in this field, recorded at ClinicalTrials.gov, are listed in Table 1. Most take into account the mutation in *IDH*, while *p53*, *RB*, *PI3K* and *RTK* modules have been neglected. Current studies mainly seek to determine whether a molecular signature respond to the chosen treatment. There are no published or current studies to date using the molecular signature of the tumor as a basis to choose treatment. This indicates that it will take a long time to have solid data in this area. Another problem is the inconsistency between studies, as they often do not confirm the findings of each other. These inconsistencies are caused mainly by the lack of standardized methods for assessing the molecular signature of tumors, the biological variability of tumors, and paucity of knowledge about the gene modules and interactions between the modules. Further studies are needed to provide simplified, standardized and clinically applicable protocols for the characterization of individual tumors, which should assist in defining the prognosis of the disease and guide the choice of treatment.

CONCLUSION

While there has been a rapid advance in our understanding of the molecular profile of gliomas, there are several challenges that impair the translation of this knowledge into clinical practice. Several markers and signaling pathways are involved concomitantly in tumor development and progression and these need to be fully elucidated for more effective therapeutic strategies. The identification of functional modules relevant to glioblastoma are promising. There is a need to standardize the methods for collection, processing, preservation and analysis of tumor samples. While MR-guided sampling improves tumor content and purity, the practicality of this method in day-to-day clinical practice still needs to be established. Results of ongoing clinical trials such as NCT04539431 and our own study should shed some light in this area in the future. It is heartening to see many clinical trials incorporating molecular markers (Table 1) in study design. The results of these studies have the potential to help develop personalized medicine strategies based on molecular profile of gliomas.

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11

Brain Mapping: Real-Time Neuropsychological Testing Experience during Low-Grade Tumor Resection

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Abstract: Awake surgery and direct electrical stimulation are performed to maximize the extent of resection while minimizing the risk of neurological and cognitive deficits. Direct electrical stimulation is a highly reliable method for monitoring simple brain functions when stimulating the sensorimotor cortex, or areas involved in speech articulation; however, negative mapping increases when testing higher functions related to language or cognition. By using DES alone, when resection involves areas supporting higher level cognition, the surgeon may receive poor feedback on the patient's cognitive status. To collect more information on the patient's cognitive status during resection, we developed real-time neuropsychological testing, an intensive neuropsychological monitoring method which is performed in addition to direct electrical stimulation. The technique includes a large number of tests that are administered in a continuously rotating and repeating pattern at different stages per anatomical area. The aim is to have a

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continuous feedback on the patient's cognitive status by reducing the risk of negative mapping. This chapter discusses this novel real-time neuropsychological test and presents the cognitive functional dynamics during surgery and recovery brought to light by the testing.

Keywords: awake surgery; brain mapping; direct electrical stimulation; plasticity; real-time neuropsychological testing

INTRODUCTION

The purpose of brain mapping with direct electrical stimulation (DES) is to monitor brain function during surgery, permitting surgeons to monitor patient's neurological functions and avoid damage to healthy brain tissue, allowing for maximum possible resection (1–4). It is highly reliable for monitoring simple functional structures such as the sensory-motor system. However, with complex brain networks, DES may not be as reliable, in part due to its on-off effect. Often, postoperative neuropsychological deficits arise. Another point worth noting is the risk of seizure when using DES, which limits the number of stimulations. During surgery, surgeons need to know with greatest possible certainty what functional consequence their actions will have. This can come from constant feedback from the patient responding throughout the resection time. In the present chapter, we describe real-time neuropsychological testing (RTNT) which is an intensive, sustained neuropsychological testing method (5) that has been developed as a complement to DES. The advantages and pitfalls of RTNT are also presented.

DIRECT ELECTRICAL STIMULATION

By using DES, a map is generated with critical points defined as "positive". An electrical stimulation triggers an effect, for instance a speech arrest, or a hand or lip movement. Three subsequent observations showing an effect at a certain site is a "positive" effect, which is sufficient for interrupting surgery. The given site with positive effect is considered functional, and if resected, could have detrimental post-surgical neurological or neuropsychological consequences. From a surgical point of view, the information about positive effect should be constant, maintaining the feedback throughout the procedure. Due to time constraints related to surgery, DES allows for short testing periods, either at cortical or subcortical levels. In addition, between two subsequent DES phases, resection continues. In that time lapse, no feedback on the patient's cognitive status is available. The negative aspect of this on-off effect is that there are frequent cases where DES does not produce a response. In such a "negative mapping" scenario, surgeons are left without any feedback. Several factors can contribute to negative mapping. Due to the focal nature of stimulation exerted, DES can electrically condition only a small and circumscribed area. The probability of obtaining a negative mapping is relatively high for cognitive functions. However, negative mapping does not always imply lack of functional role of the stimulated area and there can be deficits even after a negative response to DES.

REAL-TIME NEUROPSYCHOLOGICAL TESTING

Since 2011, the RTNT has been adopted at our Neurosurgery Unit of the Azienda Sanitaria Universitaria del Friuli Centrale as complementary to DES (5). It has been developed by our team to reduce the problems that may occur in cases of negative mapping where, as described above, a function is not detected by DES, but it does not necessarily mean that the stimulated area is not functional. The RTNT is an intensive, continuous enlarged neuropsychological testing performed throughout the surgery (Figure 1). It allows the immediate detection of neuropsychological dysfunction providing a constant and continuous functional feedback of patient's cognitive status to the surgeon, reducing the problem of missing information from DES. The test selection is based on the anatomical location. An important step is that the tests are switched from one to another very quickly to prevent a potential mismatching problem between task and anatomical point, and to maintain a time course similar to the resection times. This is a delicate phase of surgery, highly dependent on the expertise of the neuropsychologist who must be able to choose the most appropriate task for the area. The neuropsychologist has then to evaluate the performance of the patient. The baseline is constituted by the pre-surgical level of performance on the same tests (such as those tapping language, memory, visual-perception, theory of mind, executive functions, visuo-spatial transformation) that are presented also during surgery. The limit used to stop surgery temporarily or definitively coincided with a decrease of patient's performance to 70% of baseline levels.

With this approach, we observed a new feature—transitory neuropsychological dysfunction—a reduction of the performance which may completely recover to normal levels after several minutes. This sudden oscillatory scoring has never been observed while using DES alone. We argue that this pattern is suggestive of a reversible effect of mechanical tissue manipulation during surgery. Similar oscillatory scoring effects occur during intramedullary surgery (6) or surgery close to the cortico-spinal tract (7). As soon as the patient exhibited a decrement, the neurosurgeon is immediately informed and the surgeon moves resection to another part of the surgical field and return to the same point where resection altered performance after some time, if the patient has recovered.

Moreover, this method provided additional information about the intra-operative level of patients' behavior by adding a new feature that was not observed using DES alone: reversible intra-operative neuropsychological dysfunction, which consists of oscillations in the patient's responses and a richer insight into their ability to recover functionality.

RTNT comprises of a continuously repeated sequence of neuropsychological tests for lexical comprehension, picture naming, repetition, limb apraxia, short-term memory and reading. Tests are presented in a fixed order. For example, it can start with object naming, then jump to phonemic discrimination, then to word reading, and to word repetition, followed by pseudoword reading, pseudoword repetition, later to phonological discrimination, digit-span, and lastly to lexical decision, and action naming. Each of the test includes a list of 10 stimuli. Once a sequence of test is completed, the sequence is repeated, yet with a different stimulus list (Figure 2). This test repetition and alternation continues until the end of resection. When the patient completes the test at the same level as pre-surgery, resection continues (Figure 3). As soon as the neuropsychologist detects a decrement vs. the 70% threshold, the neurosurgeon is informed.

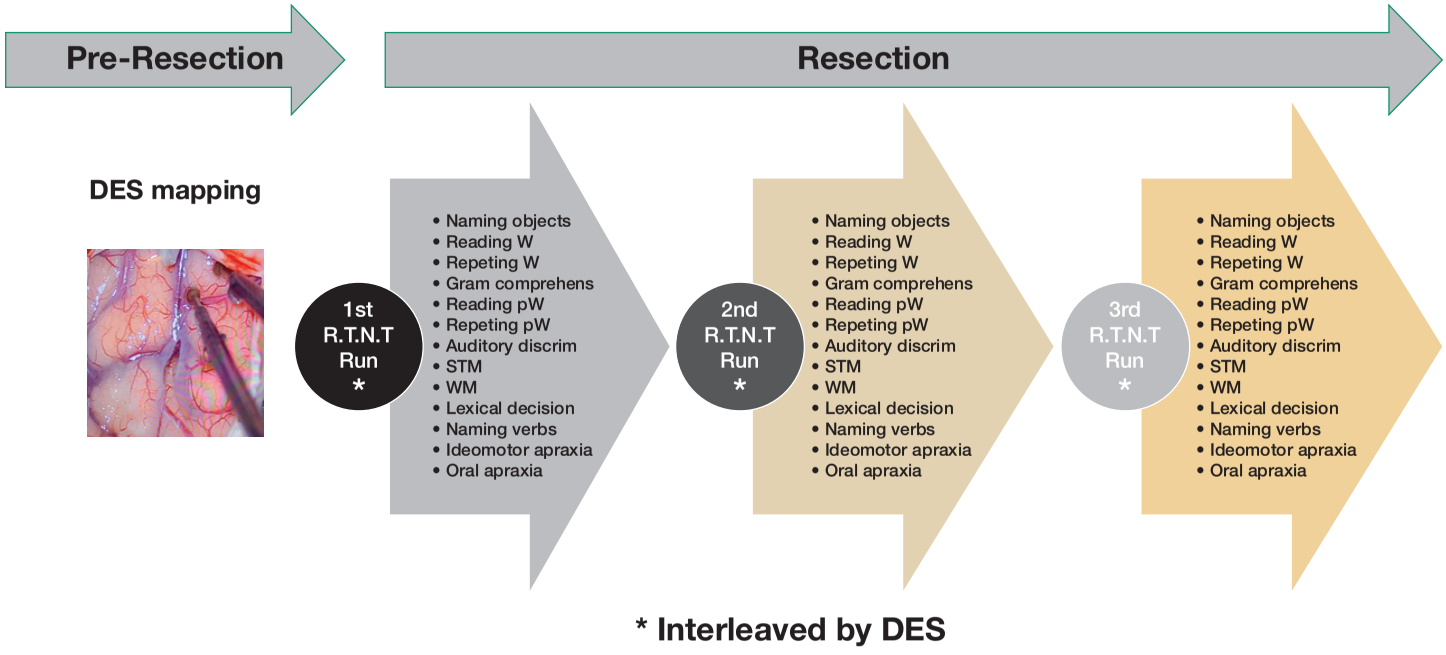


Figure 1. Sequence of events during awake surgery. The neuropsychologist uses a large number of tasks for the same area switching them quickly to reduce the risk of mismatching between test and surgical area. After craniotomy, cortical mapping is performed by direct electrical stimulation (DES) while RTNT starts at the beginning of resection and ends at the beginning of haemostasis. At intervals during the resection, DES is performed.

Data from RTNT are interpreted with respect to the patient's correct responses ranging from 0–100%, compared to their pre-surgery level (RTNT works in percentages, 100% being optimal brain function). By analyzing a preliminary series of patients, we found that an RTNT deterioration up to 70% is acceptable because it will allow the patient to make a full recovery. Once the performance is 70%, namely the patient is presented with 10 stimuli of a given test and fails 3 stimuli, performance is calculated vs. 70% of accuracy. Due to the possibility that it is just a reversible worsening of the performance, before deciding that it is a definitive value, we move surgery to another area and returning to proceed with surgery in the same area only in case the patient has recovered. At intervals, we stopped the procedure to make a further check with DES, but it was negative in the majority of cases, even at the points where a deterioration of the performance has been found.

Validation

We validated this new approach in 92 patients and the results were published in 2016 (5). RTNT has thus far been performed on a continuous series of about 300 patients and is currently in use at our Neurosurgery Unit of the Azienda Sanitaria Universitaria del Friuli Centrale. The main goal of RTNT is to preserve the patient's cognitive status. This approach has improved the extent of the resection reaching an average of 95% (range 73%–100%) while the extent of resection of a previous series of patients operated without using RTNT, which was based only on DES, information reached 90% (range 49%–100%) as shown in Figure 3C. All the data have been recorded and besides the intra-operative quick analysis we do, it has been possible to analyze them for more detailed analyses after surgery, mainly for research purposes, as presented in Figure 3A.

Left and right hemisphere

A patient undergoing a right insular resection presented with good cognitive performance throughout RTNT but experienced some neurophysiological symptoms such as strange taste and a repeated tendency to fall asleep (8). A patient with a left insular lesion maintained normal language functions but showed neurophysiological symptoms such as pain due to partial seizure arising from the temporo-/insular area as evidenced by electrocorticography (ECoG).

In the left hemisphere, we reported RTNT testing of reading abilities in a series of 49 patients (9). We found that reading performance decreased across RTNT runs in 18 patients, while accuracy remained above 70% in the majority of cases. Resections ranged from posterior, inferior and middle temporal gyrus to the temporo-parietal areas and the precentral gyrus. There was a good degree of concordance between intra- and immediate postoperative performance with a predictive value found for RTNT, and we reported spontaneous recovery of the post-surgery reading impairment at follow-up.

In the right hemisphere gliomas (10, 11), there are reports of post-surgery neuropsychological deficits. Until recently, the tendency was to operate on patients with right hemisphere lesions under general anesthesia. It is acknowledged that right hemisphere-related functions are important for maintaining a good quality

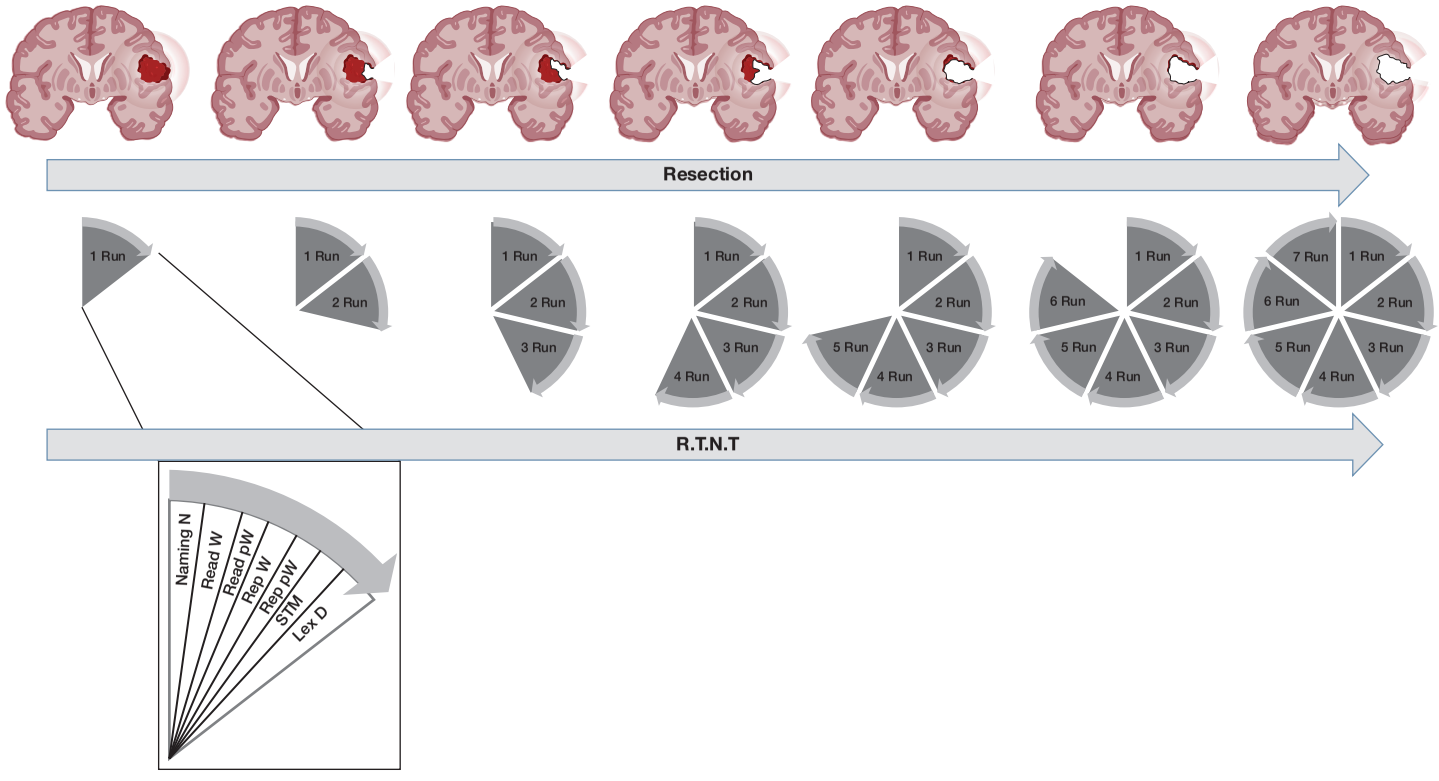
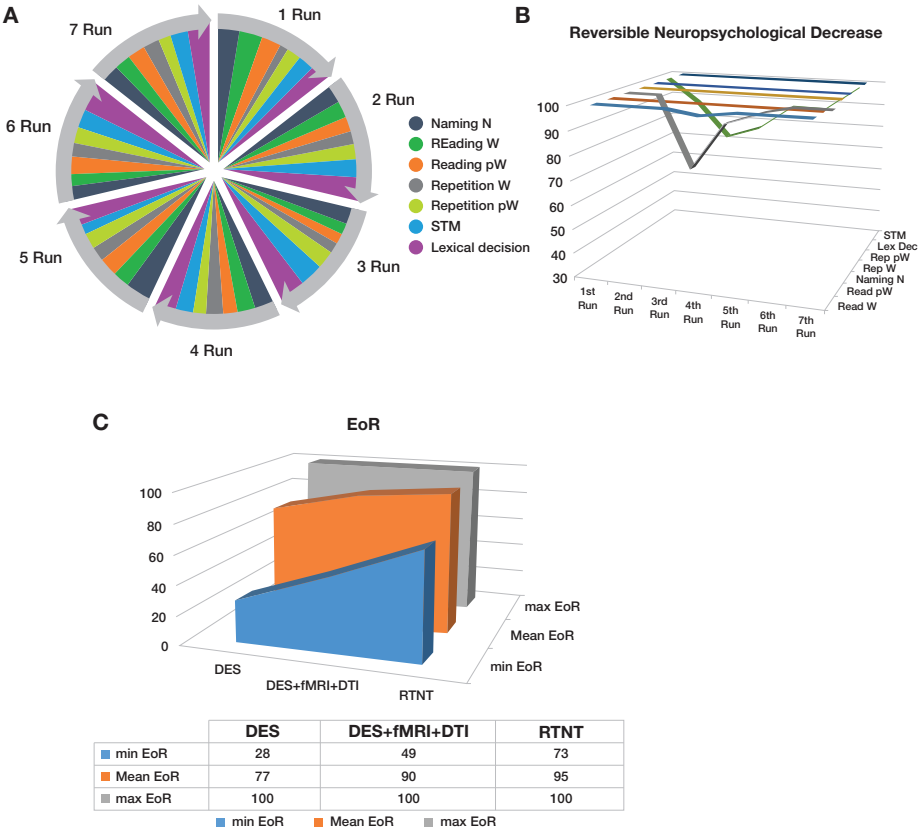


Figure 2. Illustration of how resection is carried out simultaneously with successive RTNT runs. The number of RTNT runs, namely one continuous period of testing (~1-3 min) (different runs can be compared to each other) depends on the length of resection. The task sequence included in each RTNT run is shown. Figure created in BioRender.



	DES	DES+fMRI+DTI	RTNT
min EoR	28	49	73
Mean EoR	77	90	95
max EoR	100	100	100

min EoR Mean EoR max EoR

Figure 3. Sequence of RTNT runs and list of tasks. A. The same color represents the same task which is repeated across Runs (yet with different stimuli). **B.** Patient's performance, expressed in accuracy (%), shows that for naming and lexical decision tasks there was a reversible neuropsychological decrease around the 3rd Run. We learnt recognizing and detecting reversible neuropsychological deficit patterns, which are often followed by recovery, allowing to proceed with the resection. A positive DES would simply have stopped resection. **C.** The extent of resection over time evolved and increased when RTNT was introduced.

of life and should be preserved during awake surgery when possible (12). Intraoperative monitoring of higher functions related to the right hemisphere include visuospatial cognition, socio-cognitive functions and executive functions which are still poorly documented and remain an open issue. We adopted four RTNT protocols for the frontal and parietal lobes, the temporo-insular region and premotor area, except for the motor sensory function. Our experience with RTNT in the right hemisphere involves 103 patients. The most frequently used task in patients with lesions involving the visuo-spatial network is the Verbal Milner Landmark task (13) (Figure 4A) which is used to monitor visuo-spatial functions and detect the first signs of neglect. In this task, the patient is required to decide which part of a segment colored in black and red is longer (for half of the items)

or shorter (for the remaining of the items). The mean accuracy achieved by this group of patients is 80.55%. In summary, RTNT is currently used in selected cases for awake surgery in the right hemisphere as it enables us to try and extend resection based on feedback of a proficient performance.

A new feature we observed during RTNT is behavioral/emotional reactions. In an exploratory analysis performed on 100 patients, we found that these were most frequently observed (Mann-Whitney Test, $Z = -1.97$, $p < .05$) in patients with right hemisphere lesions compared to patients with left hemisphere lesions. Behaviors or neurophysiological manifestations included panic, complaining, crying, pain, nausea, apathy, anger, agitation, sweating, and excessive tiredness (Figure 4B). We observed that often lesions in such patients are localized in the temporo-insular area (Figure 4C). This did not change the surgical strategy.

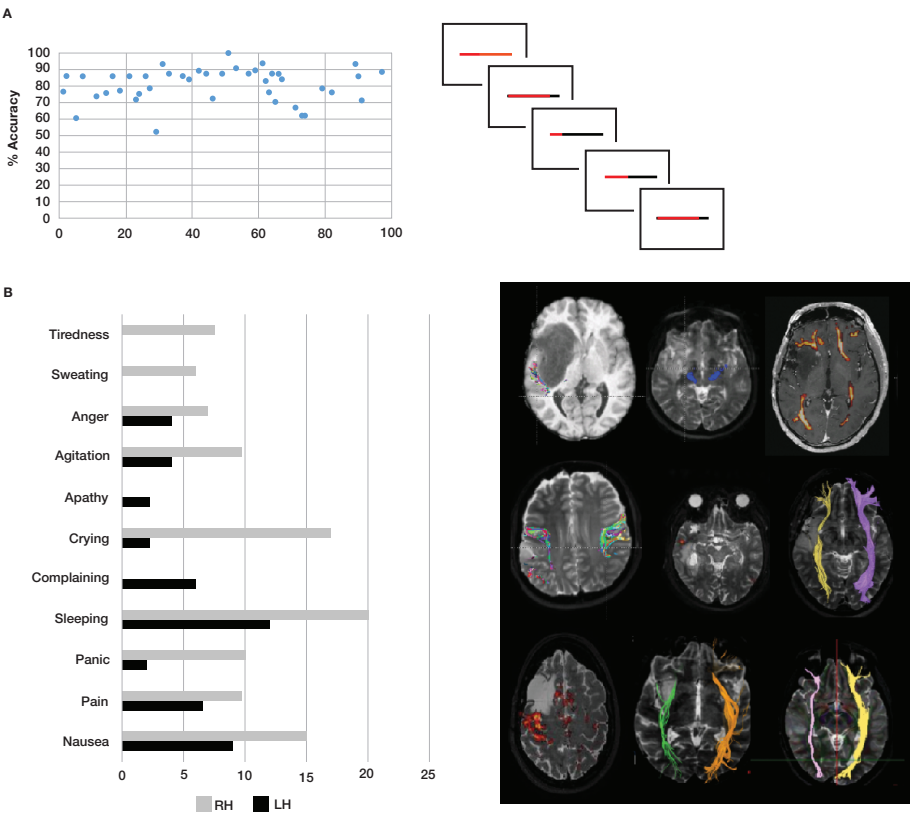


Figure 4. Patients' overall accuracy. A. Milner Landmark Test, and an example of the different items with shorter (black) or longer segments (red). **B.** Patients (%) showing behaviors or neurophysiological manifestations, and examples of the lesion localization in patients with right hemisphere lesions showing such phenomena.

Recurrent surgery

We retrospectively examined a series of 40 patients undergoing recurrent surgery in the left hemisphere, mainly in the left insular cortex and in the left inferior frontal gyrus (14). The study first compared the pre- and post-surgical neuropsychological status after first and second surgery. We found no significant difference in the number of patients scoring within the normal range. Their level of performance did not differ statistically either. In the same study, we addressed the patients' neuropsychological evolution from pre- and post-resection assessments of the initial and the recurrent surgery and found no significant difference in the number of patients scoring within the normal range. In addition, we analyzed their level of performance, which was stable for all tasks except that for phonological fluency. Based on these results, we argued that repeated glioma surgery is possible without major neuropsychological sequelae.

Young patients

The RTNT approach has been adapted to awake surgery for young patients. We developed the Junior Real Time Neuropsychological Testing (j-RTNT) for young patients as neuropsychological tests differ from those used with adults. We included neuropsychological tasks related to the area to be resected; the tasks were taken from the neuropsychological assessment for preschoolers, children, and adolescents (Nepsy-II), creating different RTNT runs (15).

Advantages and disadvantages of RTNT

The RTNT approach offers the possibility to obtain a continuous and reliable feedback about the patient's cognitive status, through all the resection phases. The test administration occurs continually, while DES is discontinuously performed. RTNT is not subjected to the limits of a focal stimulation like DES, thus avoiding the risk of negative mapping. In our experience, RTNT allows a quicker resection, since the surgeon continuously receives feedback on the patient's status. In addition, by using the RTNT approach we detected a new neuropsychological feature, that is the reversible neuropsychological decrease, and we learn by experience that in the majority of the cases, resection could continue. In contrast, a positive DES would stop resection. As a consequence, the extent of resection could be different by using the two approaches. RTNT is designed to include a large number of tests and stimuli, and this property allows a higher confidence and a proper match between an area and the test. By analyzing the patients' follow-up neuropsychological tests, we could learn the most reliable test to be used intra-surgery for a given area, those who fully recover in the post-surgical phase, and those who are more resistant to recovery. We also gained a better knowledge about the tasks that are useful during surgery in the right hemisphere and in general, a better insight in understanding higher level cognition during surgery. Lastly, RTNT made it possible to increase the average extent of resection and the delivery of a continuous feedback on the patient's cognitive status enabling surgeons to achieve gross total (and supra-total) resections.

The disadvantage of using the RTNT approach is that demanding levels of collaboration and flexibility are required. This can limit the number of tests that can be used intra-surgery. In addition, a high neuropsychological expertise is also required.

CONCLUSION

The first prerequisite in glioma surgery, particularly for low-grade gliomas, is to remove the tumor as extensively as possible to improve prognosis. Low-grade gliomas suited for surgery are generally located supratentorially involving the cortex and the white matter beneath. Apart from simpler functions, meaning motor and somatosensory functions, which actually are represented in a very limited part of the central area, the remaining cortical areas and the white matter are involved in cognitive functions of varying complexity. These higher cognitive functions are based on complex network systems and as seen clinically, are characterized by high inter-individual variability (16–18). The only way to monitor these functions is neuropsychological testing—aware that the anatomo-functional correlations are often limited with respect to the microsurgical spatial resolution. It is only by using a large number of tests that we can avoid mismatches with the anatomical localization and have a relatively safe functional feedback of the area under resection. Our experience indicates that real-time testing needs patients to be highly collaborative, and this is the only limitation of this approach. This method is demanding for both the neuropsychologist and the patient. For each individual patient, the greater the collaboration during these tests taking place one immediately after the other, the higher the quality of the feedback indicating whether surgery is progressing safely. This approach also offers higher prediction on the recovery of higher cognitive functions.

Conflict of interest: The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this chapter.

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12

Pathogenesis and Management of Brain Tumor-Related Epilepsy

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Abstract: Up to 50% of patients with brain tumors will initially present with seizures, while an additional 10–30% will develop seizures during the course of the disease. Gliomas are the most common primary intracranial tumors and are associated with a number of changes which are involved in the pathogenesis of epilepsy, including blood-brain barrier disruption, molecular changes, edema, and peritumoral environmental changes. Epilepsy is a source of significant morbidity and mortality for patients with gliomas. The two main treatments for patients with glioma-related epilepsy involve antiepileptic drugs as well as surgical resection of the mass and surrounding epileptogenic tissue, if feasible. Given the propensity for neighboring tissue to also be epileptogenic, intraoperative electrocorticography can be of benefit to define the seizure onset and spread areas. Surgical treatment of glioma-associated epilepsy can provide significant relief for affected patients. Unlike non-lesional epilepsy, which is primarily managed medically, glioma-related epilepsy frequently requires surgery because of its medically refractory nature.

Keywords: anti-epileptic drugs; high-grade gliomas; intractable epilepsy; refractory seizures; tumor-associated epilepsy

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INTRODUCTION

Seizures are one of the common presenting symptoms of brain tumors, accounting for up to 50% of initial presentations. One third of patients diagnosed with a brain tumor will develop seizures during the clinical course if they did not initially present with seizures (1–4). Although the incidence of seizures is high among brain tumor patients, the incidence of intracranial tumors as an underlying etiology of epilepsy is relatively low (3, 5). Despite significant advances, management of seizures in patients harboring a brain tumor remains challenging. These difficulties arise from suboptimal response to anticonvulsants, interplay between antiepileptic drugs (AEDs) and chemotherapeutic agents, and possible adverse effects of both medical and surgical treatment. Seizures can tremendously affect patients' quality of life and negatively impacts overall survival (6). The focus of this chapter will be new literature and guidelines related to brain tumor-related epilepsy (BTRE).

EPIDEMIOLOGY AND PATHOGENESIS

The exact pathophysiology of BTRE is not well characterized; however, it is thought to be multifactorial (Figure 1) (7, 8). Tumor burden, type, location, growth rate, microenvironment of the blood-brain barrier, altered neurotransmitter homeostasis, and gap junction alterations are factors that influence

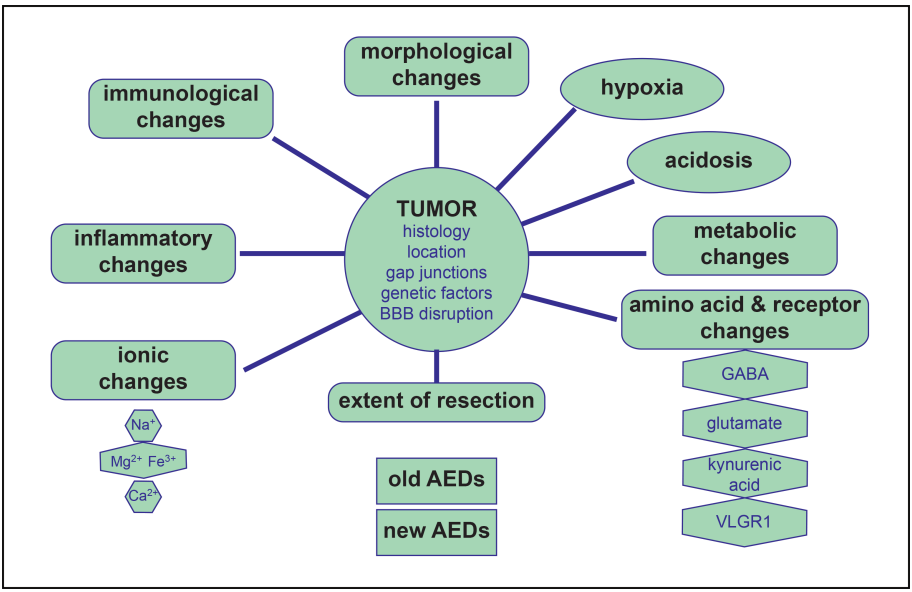

Figure 1. Known mechanisms underlying the pathogenesis of brain tumor-related epilepsy. Modified from Wu et al. (39).

BTRE (7, 8). The likelihood of epilepsy among patients with brain tumors differs depending on the tumor's histopathological subtype. Patients with low-grade gliomas have a greater tendency to suffer from seizures than those with high-grade gliomas. One study found that patients diagnosed with low-grade glioma had a significantly higher rate of seizures compared to patients diagnosed with glioblastoma (85% vs. 49%, respectively) (9). Dysembryoplastic neuroepithelial tumors (DNETs) and gangliogliomas have an extremely high propensity for seizures with an incidence greater than 80% (3, 10). Metastatic lesions tend to have a low incidence of seizures (9, 11, 12). Melanoma, however, has the highest seizure rate among the metastatic lesions because it involves gray matter, frequently has multiple lesions, and has an intrinsic high frequency of hemorrhage (13, 14).

Tumor Location

Tumor location is one of the most important aspects to consider when it comes to tumor epileptogenesis. Cortical tumors involving the frontal, temporal and parietal cortices as well as tumors in the cortical gray matter are associated with greater seizure frequency compared to lesions involving the infratentorial region, suprasellar region, or occipital lobe (Table 1) (1, 10). Seizure type is also associated with anatomical location of tumor. For example, focal awake seizures are associated with lesions involving left inferior and middle frontal gyrus, while focal unaware seizures are associated with the right temporal-insular region (15).

Tumor Growth Rate

Studies show that seizure prevalence and tumor growth rate are inversely proportional (9). Intuitively, slower growing and more indolent gliomas have a longer amount of time to provoke a seizure. Moreover, epileptogenesis requires complex re-organization and vascularization of tumor cells which often does not happen with rapidly growing tumors (7). Moreover, slow growing neoplasms tend to possess innate epileptogenic properties (7).

The pathogenesis of epilepsy differs among the various types of tumors. Tumors such as DNETs have high incidence of seizure because they tend to cause

TABLE 1 **Seizures by tumor location in 65 patients with gliomas. Adapted from Lote et al. 1995 (9)**

Tumor Location	Seizure at Onset	Recurrent Seizures	Late Onset Seizures	Status Epilepticus
Frontal	10/19	8/10	2/9	0/19
Temporal	3/11	3/3	2/8	1/11
Parietal	10/24	7/10	3/14	5/24
Occipital	0/2	–	2/2	1/2
Multifocal/Bilateral	6/9	3/6	1/3	3/9

cortical disruption due to disruption of the underlying cortical and subcortical structures (3, 10). The mechanisms behind the higher seizure frequency of other low-grade lesions is likely secondary to mechanical and vascular changes which slowly develop overtime (10). In contrast, high-grade gliomas and other rapidly dividing tumors tend to cause seizures because of irritation from necrosis or products of hemorrhage, such as hemosiderin (10, 16–21).

Neurotransmitters and Gap Junctions

Various animal and human tissue studies have identified glutamate, γ-aminobutyric acid (GABA), and adenosine kinase (ADK) as possible contributory factors for epileptogenesis in patients with brain tumors (22–24). Glioma studies in animals illustrated that seizure activity originated due to elevated glutamate production causing hyperexcitability around the peritumoral area (22). Studies comparing patients with lesion-associated medically refractory epilepsy and patients with similar lesions but no clinical epilepsy demonstrated that approximately 73% of tissue obtained from patients with lesional refractory epilepsy shows disruption of GABA and N-methyl-D-aspartate (NMDA) receptors (19). Dysregulation of ADK among the peritumoral tissue has also been hypothesized to induce seizure activity among patients with brain tumors. One study comparing normal brain tissue with excised epileptogenic foci of patients with epilepsy showed higher expression ADK in tissue of epilepsy patients (23). Disruption of the blood-brain barrier can also cause dysregulation of neurotransmitters such as glutamate and GABA and can contribute to BTRE (25). A recent study noted that proteolytic enzymes released by tumor cells disrupt perineuronal nets resulting in decreased GABAergic inhibition and overall excitation/inhibition imbalance (26).

One of the main functions of gap junctions in the brain is intercellular communication. Connexin 43 (CX43) is an important transmembrane protein and functional element of gap junction; its expression was found to be high in glial cells, such as astrocytes (27). Peritumoral cells in low-grade gliomas also express CX43 to a greater extent than the peritumoral cells of high-grade gliomas, which could be a reason why patients with low-grade gliomas have seizures more frequently than patients with malignant gliomas (28–31). Unsurprisingly, drugs that target gap junctions may have an anticonvulsant effect (30, 31).

Molecular Genetics and Peritumoral Environmental Changes

The genetic implications of BTRE are poorly understood. Genes such as *LGI1* (a tumor-suppressor gene) and phosphatase and tensin homolog (PTEN) have been associated with gliomas and epilepsy; however, their exact role in epileptogenesis is not well characterized (32–35). The microenvironment and neurotransmission between peritumoral tissue and normal brain tissue is vastly different (36). Gliomas cause disruption of the blood-brain barrier in surrounding tissue by changing the endothelial permeability which can lead to vasogenic edema, inflammatory changes, poor perfusion, and changes in hemostasis (37). All these microenvironmental changes in peritumoral tissue can lead to sodium and calcium imbalance in the neuronal cells eventually causing hyperexcitability and seizures (19, 20, 38, 39).

CLINICAL PRESENTATION

It is well documented that BTRE causes significant burden in quality of life, mental status, cognition, and morbidity (40–43). The clinical manifestation of seizures related to brain tumors are usually focal or generalized with motor onset. Focal seizures are mostly location-dependent and correspond to specific function. For example, involvement of the precentral gyrus will typically manifest as focal motor seizures involving the contralateral extremities. Visual changes, altered mental status, behavioral changes, or altered sensorium could also be clinical symptoms associated with tumor-related seizures. Patients can also experience postictal Todd's paralysis, severe agitation with psychosis, and status epilepticus (SE) (44). The rate of SE in patients with brain tumors is variable; however, approximately 7% of all SE cases can be attributed to brain tumors (45). Patients who suffer from SE and brain tumors have higher 30-day mortality when compared patients with SE who do not have brain tumors (46). As mentioned above, patients with low-grade gliomas are more likely to have seizures compared to patients with high-grade gliomas (9). Additionally, patients with low-grade gliomas are more likely to have secondary generalized seizures. Focal aware seizures are more common among patients with high-grade gliomas (47).

MEDICAL MANAGEMENT OF BTRE

Epilepsy can be defined as at least two unprovoked seizures occurring more than 24 hours apart or one unprovoked seizure with at least 60% probability of another one occurring over the next 10 years. With this definition, any patient with a brain tumor who has one seizure will automatically have epilepsy (48). As a result, it is imperative to treat these patients with AEDs to prevent seizures and their complications.

The American Association of Neurology (AAN), the Congress of Neurological Surgeons (CNS), and the American Society of Therapeutic Radiology and Oncology (ASTRO) all recommend withholding AEDs in brain tumor patients who have not had a seizure. In an instance where an AED has been started, it is recommended to withdraw after the first week of surgery (4). BTRE patients, however, need AEDs to prevent further seizures. AEDs such as levetiracetam, lamotrigine, lacosamide, topiramate, and pregabalin are recommended as they have favorable side effect profiles (49, 50). In a retrospective study comparing seizure control rates and adverse effects of levetiracetam and valproic acid (VPA), both AEDs show similar seizure control rates. VPA had a statistically significant higher rate of adverse drug effect when compared to levetiracetam (51). Another study demonstrated that patients with BTRE and high-grade gliomas tended to require multiple AEDs for seizure prophylaxis (47). VPA or a combination of VPA and levetiracetam had more success in controlling seizures than other agents (47).

Drug–drug interactions present some additional challenges in patients with BTRE who are taking multiple medications, including one or more AEDs with or without chemotherapy. AEDs that are metabolized in the liver have the most interactions with other drugs. Phenobarbital, carbamazepine, oxcarbazepine, and phenytoin are classically known for their enzyme inducing abilities, allowing faster

metabolism of chemotherapy drugs such as methotrexate, steroids, paclitaxel and so on, potentially compromising the efficacy of oncological treatment (52).

Monotherapy is preferred when it comes to BTRE as it safer for the patient and compliance is less of an issue. Nonetheless, patients having seizures refractory to AED monotherapy will require additional agents. This is more common among patients with BTRE. In a study of 99 patients with BTRE, more than half did not respond to one AED. Among the non-responders, VPA and levetiracetam was the most effect combination to prevent further seizures (47). Studies have demonstrated that more than 50% of patients continue to have seizures despite the maximal medical management (1, 53). Seizures refractory to two AEDs will likely not be controlled with additional medications (54).

Surgical Treatment

Surgery is essential for diagnosis and treatment of brain tumors. In patients with BTRE, surgery is required for tissue diagnosis, reduction of tumor burden and mass effect, as well as seizure management. One study demonstrated that two-thirds of the epileptogenic focus of patients with BTRE is located within or adjacent to the tumor (55). Thus, surgical intervention can often be curative for patients with BTRE (56). In patients with BTRE who failed medical management with two first line AEDs, surgery can be beneficial for seizure control (4, 54, 57). In one series of 207 patients, 82% of patients with BTRE were seizure-free following tumor resection (56). This study also demonstrated that patients with one seizure focus tended to have better outcomes than patients with multiple seizure foci (56). A meta-analysis involving 773 patients with BTRE who underwent surgical resection showed approximately 71% were seizure-free after surgery (57, 58). The authors also demonstrated that patients who underwent gross total resection of the tumor had higher seizure freedom rates (58). As previously described, DNETs and gangliogliomа have higher frequency of seizures at presentation and they can be especially resistant to anticonvulsants. Thus, these patients often require surgical treatment (40, 55).

Surgical planning for medically refractory epilepsy starts with obtaining a regular scalp EEG to localize the seizure focus; however, these non-invasive studies are generally not adequate for precise seizure localization. Intracranial EEG with subdural grids, strip electrodes, and depth electrodes can be extremely helpful to accurately localize seizure focus and provide better outcome for patients with BTRE (59, 60). Electrocorticography (ECoG) and stereoencephalography (SEEG) are techniques utilized to further help localize the seizure focus when scalp EEG is inconclusive or unclear.

Different surgical treatment options as well as advanced imaging modalities are available for patients with BTRE. Intraoperative cortical brain mapping with electrocorticography, radiosurgery, and laser interstitial thermal therapy are additional surgical techniques that can be effectively utilized in BTRE. EEG mapping is also another modality that can be beneficial for identifying the epileptogenic focus. Epileptogenic foci can be identified within or overlying the tumor, the peritumoral tissue, and even distant areas away from the tumor (Figure 2) (61, 62). The extent of tumor resected directly correlates with seizure freedom; however, patients may benefit from subtotal resection if the epileptic focus was identified before the resection, especially if the tumor is in eloquent areas of the brain (62).

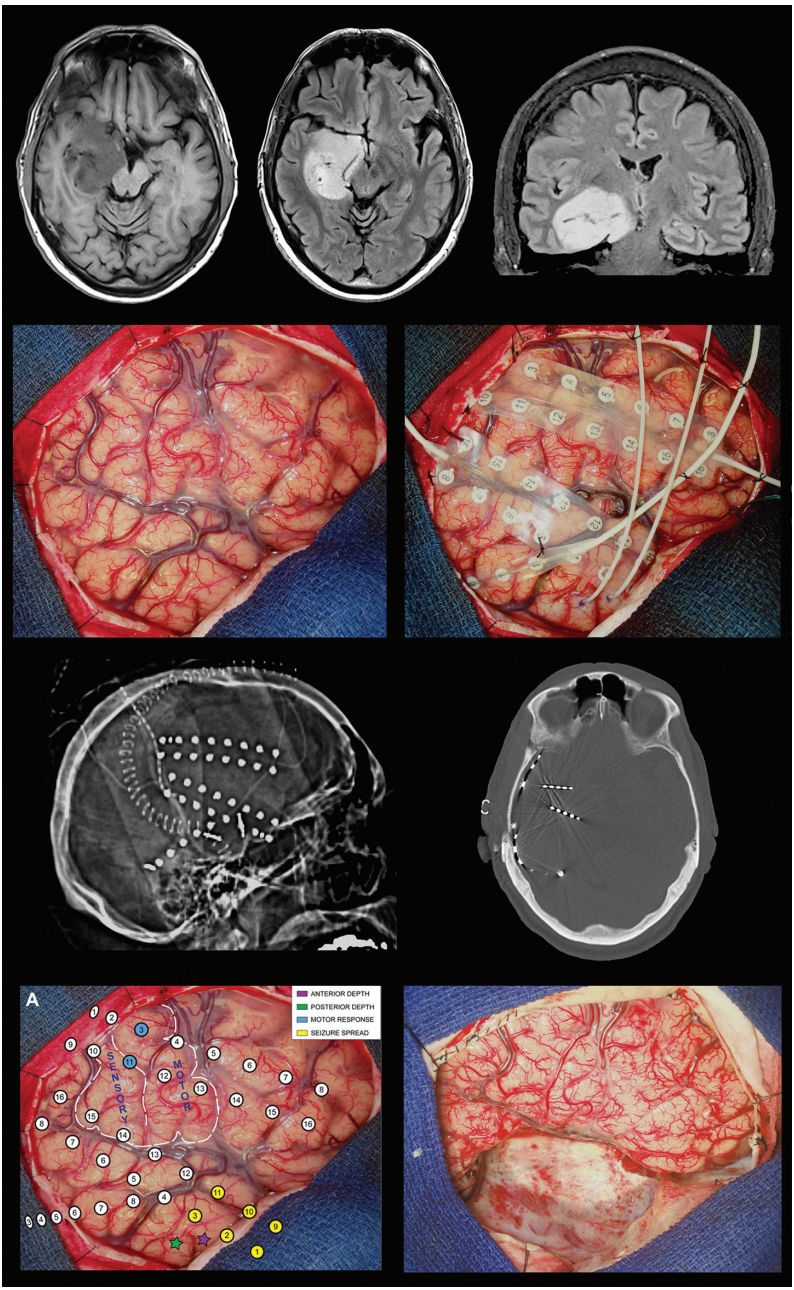

Figure 2. Surgical management of brain tumor-related epilepsy. This patient presented with recurrent seizures and a non-enhancing mass in the right medial temporal region (*top row*). Intraoperative photographs during intracranial electrode implantation (*second row*). Postsurgical CT scan showing placement of subdural grid electrodes and depth electrodes (*third row*). Intraoperative photographs showing intracranial electrode arrays with cortical mapping results and following resection of the tumor and epileptogenic tissue (*bottom row*).

It is essential to identify patients where the tumor is not the primary epileptogenic focus, as they may benefit from a combined approach encompassing both tumor resection and epilepsy surgery (61).

Gross total resection can provide seizure freedom as high as up to 87% compared to 55% seizure freedom with subtotal resection (63). Seizures in BTRE patients are best treated surgically irrespective of AEDs (40). It is also particularly important to note that surgery has its own risks. Proper discussion and informed consent with patient and family (if applicable) regarding risks, benefits, and alternatives to surgery is essential. Intractable epilepsy associated with brain tumors can significantly impact a patient's life. Studies suggest that early surgical resection is beneficial for disease control and improvement in quality of life (4, 56, 63, 64). Equivalent results were noted when compared to extent of surgical resection and seizure freedom (58, 63, 65–67).

CONCLUSION

Among patients with brain tumors, seizures are one of the common presenting symptoms (10). Many studies have shown that tumors have intrinsic effects on surrounding normal brain tissue, causing it to become epileptogenic. As we discussed, pathogenesis of BTRE involves multiple factors such as tumor size, location, types of tumor, growth rate, peritumoral environmental changes, and much more which is still to be discovered. There is no evidence for AEDs as prophylaxis for brain tumor patients without seizures. However, in patients with BTRE, first- and second-generation AEDs are both beneficial medical treatment options. A carefully planned surgery can help patients with BTRE achieve complete seizure freedom and cytoreduction. With advances in EEG mapping technology and targeted therapies against tumors, a comprehensive multidisciplinary management approach should be undertaken and can help improve quality of life as well as long-term oncologic and seizure outcomes in patients suffering from brain tumor associated epilepsy.

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13

Glioneuronal Tumors: Insights into a Rare Tumor Entity

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Abstract: Glioneuronal tumors are a group of rare neoplasms made up of neural and glial components in heterogenous proportions, generally exhibiting WHO grade I clinical behavior. These tumors affect infants, children and young adults, but are also described in adults and the elderly. They are strongly associated with seizures. Tumor subtypes described under the umbrella of glioneuronal tumors are actively evolving but to date comprise central, extraventricular and lipo- neurocytoma, desmoplastic infantile astrocytoma and ganglioglioma, diffuse leptomeningeal glioneuronal tumor, dysembryoplastic neuroepithelial tumor, papillary glioneuronal tumor, rosette-forming glioneuronal tumor of the fourth ventricle, rosetted glioneuronal tumor with neuropil-like islands, gangliocytoma, ganglioglioma, anaplastic ganglioglioma and paraganglioma. They vary in radiographic appearance, with some exhibiting large heterogenous solid/cystic masses. With large scale genetic and molecular analyses ongoing, classification continues to evolve. Seizure management and surgical resection represent the cornerstones of management, with the use of systemic agents and radiation lacking conclusive results. Optimal management requires multidisciplinary discussion including

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neuro-oncological and neuro-surgical expertise due to both the rarity of these tumors and the lack of evidence with data confined to small retrospective series and reviews.

Keywords: desmoplastic infantile astrocytoma and ganglioglioma; dysembryoplastic neuroepithelial tumor; glioneuronal tumors; neurocytoma; late effects

INTRODUCTION

Glioneuronal tumors are rare tumors comprised of both neural and glial components present in heterogenous proportions displaying indolent WHO grade I behavior (1–3). More recently, molecular characterization has allowed for more robust classification (4–9). The subtypes falling under the umbrella of glioneuronal tumors are actively evolving but currently include: central, extraventricular and lipo- neurocytoma; desmoplastic infantile astrocytoma and ganglioglioma (DIA/DIG), diffuse leptomeningeal glioneuronal tumor, dysembryoplastic neuroepithelial tumor (DNET), papillary glioneuronal tumor (PGNT), rosette-forming glioneuronal tumor of the fourth ventricle (RGNT), rosetted glioneuronal tumor with neuropil-like islands (GNTNI), gangliocytoma (GC), ganglioglioma (GG) and anaplastic ganglioglioma and paraganglioma (1, 2). Evolving entities including low grade neuroepithelial tumor of the young (9), multinodular and vacuolating neuronal tumor (10), and entities such as paraganglioma (11), are not described in detail in this chapter. Depending on the subtype, glioneuronal tumors can occur in all age groups. Most glioneuronal tumors present with seizures, depending on location and rate of growth. Patients can present with increased intracranial pressure, acute neurological deficits, hydrocephalus, diffuse leptomeningeal spread and symptoms of cord impingement or compression. Overall, the data surrounding glioneuronal tumors remains scant, largely comprised of small retrospective series and reviews of literature (1–3). Management involves seizure and symptom control, including resection when possible (12, 13). In sub-totally resected disease and recurrent or grade II/III tumors, adjuvant treatment in the form of radiation and or chemotherapy may be administered; however, data on improvements in outcome are lacking (14–21). Due to the indolent behavior of these tumors, late effects and survival are increasingly being examined (22–24).

CLINICAL PRESENTATION

Over 50% of patients present with headache (20), hydrocephalus (21, 25) and focal neurological deficits. Glioneuronal tumors are by far the most common histological type of brain tumors requiring surgery for epilepsy management (26) and are therefore part of the “low grade epilepsy associated neuroepithelial tumors” family (26–29). Patients can present with seizures in infancy, childhood or early adulthood and most patients will have a mean duration of epilepsy of approximately 5 years with a range of 0.1 to 35 years (8). A family history of seizures or brain tumors is not typically reported on history and most patients

exhibit multiple seizure types with seizures that tend to be drug-resistant. About 42.9% of patients display two or more types of seizures with more patients presenting with complex partial seizures (dialeptic seizure, with vegetative or affective aura, psycho-sensorial aura and automatisms) or partial seizures evolving to secondarily generalized seizures (8). Temporal location is common, and the seizure presentation can be associated with focal cortical dysplasia (6). Although previous publications indicated a possible predilection for male (8) or female gender (1, 14, 30), more recent data suggests a 1:1 distribution (5, 22). Age at presentation for DIA/DIG is infant to 33 years while for other types such as PGNT, RGNT and GNTNI, it is 12–70 years (7, 8, 20). In addition to seizures, patients can present with hydrocephalus (21, 25), increased intracranial pressure as well as focal neurological deficits depending on tumor location, rate of growth and age. DIA/DIG presents usually, although not exclusively, in infants with increasing head circumference and seizure (31). Most commonly, patients with low-grade tumors present with headache, nausea, vomiting, and seizure; less frequent presentations include neurological deficits, loss of consciousness and chronic intermittent microhemorrhages (32–34) (Table 1).

Pathological, molecular and imaging features

Glioneuronal tumors have been reported since 1910, with the number of publications growing in the last 10 years as the identification of molecular alterations has allowed for better differentiation of glioneuronal tumors from other similar tumors in differential diagnosis (Figure 1, Table 1). Previous limitations in diagnosis were related to limited ability to elicit radiographic differences, scant material for pathological analysis and the overall rarity of glioneuronal tumors. Ganglioglioma, paraganglioma, central neurocytoma and DNET have been reported on for some time (Figure 1) (1–3, 35–44). RGNT (39–41), PGNT (42, 43) and GNTNI (44) were added to the WHO classification in 2007 (1). In 2016, the classification

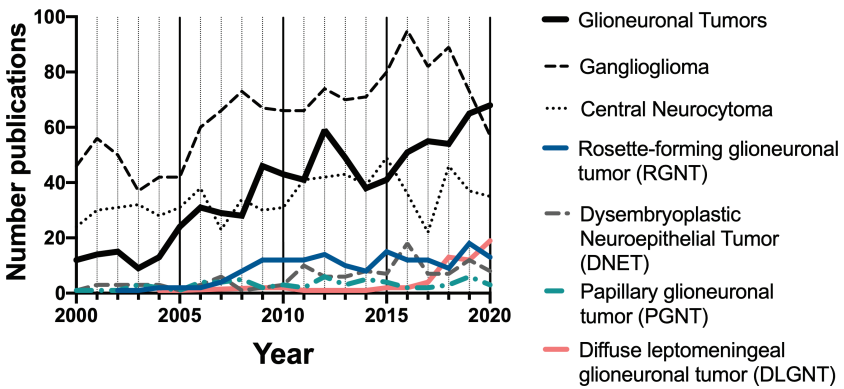

Figure 1. Number of publications from 2000 to 2020 by major glioneuronal tumor histologies. Author's own analysis based on literature search information. Retrieved October to November 2020 from https://pubmed.ncbi.nlm.nih.gov/?term=glioneuronal+tumors&sort=date (35).

TABLE 1 **Summary of glioneuronal tumor features**

Subtype	Age group	Presentation	Location/Imaging	WHO grade	Genetic/Molecular Features	Management
Central neurocytoma/ Extra-ventricular Neurocytoma	Young adults	Increased intracranial pressure	Lateral ventricle/third ventricle (central neurocytoma)	II	DNA copy alterations	Surgical Resection Radiation (if Subtotal resection)
Liponeurocytoma	Young to older	Increased intracranial pressure	Cerebellum	II	TP53 mutation	Surgical Resection (recurrence common) Radiation
Desmoplastic infantile astrocytoma and ganglioma (DIA/ DIG)	Infants	Increasing head circumference Seizures	Large Solid/cystic mass	I	RAF V600E NRTK-1 Fusion	Surgical Resection
Diffuse leptomeningeal glioneuronal tumor (DLGNT)	Children Young adults	Hydrocephalus Cord compression Seizures	Leptomeningeal tumors/ spinal mass	I	BRAF gene fusion, 1p19q co-deletion (no IDH mutation) H3K27M described	Resection Chemotherapy and Radiation when recurrent
Dysembryoplastic neuroepithelial tumor (DNET)	Children Young adults	Epilepsy	Temporal lobe Hyperintense on T2 MRI and FLAIR Multicystic	I	PIK3CA/FGFR1 mutations	Epilepsy management Surgical Resection
Gangliocytoma (GC)	Children	Epilepsy	CNS (no specific location)	I	Lhermitte-Duclos Disease (Dysplastic cerebellar gangliocytoma PTEN/ SDHB)	Epilepsy management Surgical Resection

Table continued on following page

TABLE 1 **Summary of glioneuronal tumor features (Continued)**

Subtype	Age group	Presentation	Location/Imaging	WHO grade	Genetic/Molecular Features	Management
Ganglioglioma (GG)	Very young to very old	Epilepsy	Predilection for temporal lobe Cystic and can be contrast enhancing	I	BRAF V600E H3K27M described	Epilepsy management Surgical Resection
Anaplastic ganglioglioma	Children and adults	Epilepsy Acute neuro symptoms	Similar to GG Contrast enhancing	III	BRAF V600E	Surgical Resection Chemotherapy and Radiation
Papillary glioneuronal tumor (PGNT)	Young adults	Epilepsy	Supratentorial Large Solid/cystic mass	I	SLC44A1-PRKCA NOTCH1-PRKCA	Surgical Resection
Rosette-forming glioneuronal tumor of the fourth ventricle (RGNT)	Young adults	Epilepsy	Fourth ventricle	I	PIK3CA/FGFR1 mutations IDH1 mutation	Surgical Resection
Rosetted glioneuronal tumor with neuropil-like islands (GNTNI)	Young adults	Epilepsy Acute neuro symptoms	Solid/cystic mass Enhancing	II/III	Evolving	Surgical Resection Chemotherapy and Radiation when possible

added diffuse leptomeningeal glioneuronal tumors (DLGNT) (1, 2, 45–51) where the number of published case reports, pathological studies and reviews has grown since (Figure 1) (35).

Pathology

The common feature of glioneuronal tumors is the presence of both glial and neuronal tissue as evidenced by glial fibrillary acid protein- positive cells and synaptophysin-positive neuronal cells forming solid areas (Figure 2A (52)). The rarity of PGNT (Figure 2A (52); 68 cases in literature), RGNT (Figure 2B (53); 130 cases in literature), ganglioglioma (Figure 2C (54); 1.3% of all primary brain tumors), neurocytoma (Figure 2D (55); 0.1–0.5% of all brain tumors), makes diagnosis difficult (1–4). While each glioneuronal tumor can display certain distinct features (Figure 2), in some instances a tumor may demonstrate overlapping histologic features with mixed components (36) making diagnosis challenging. With the exception of central neurocytoma (WHO grade II), extraventricular neurocytoma (WHO grade II), liponeurocytoma (WHO grade II), GNTNI (WHO grade II/III) and anaplastic ganglioglioma (WHO grade III), glioneuronal tumors

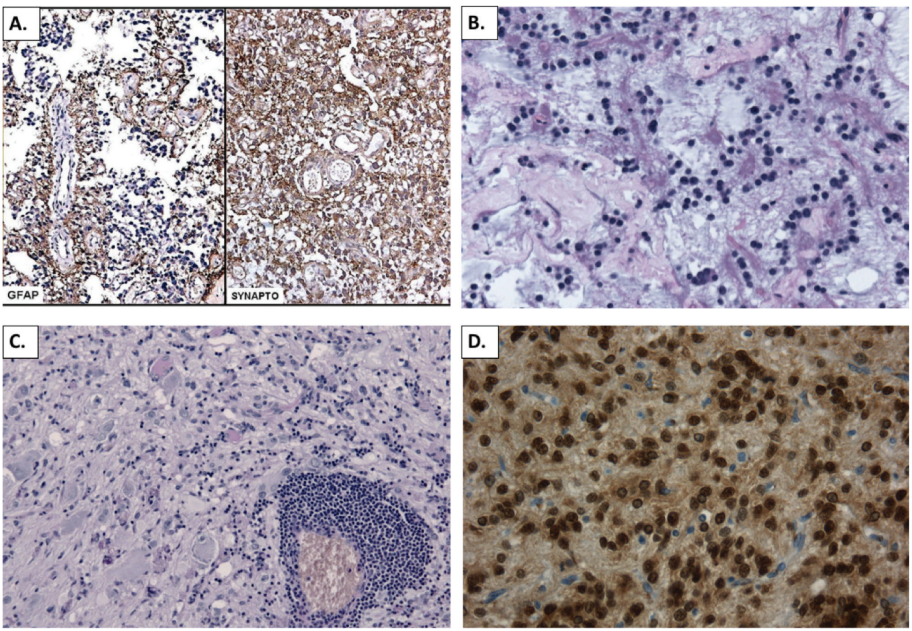

Figure 2. Pathologic features of glioneuronal tumors. A. Histopathology of papillary glioneuronal tumor (PGNT): the pseudopapillae formed by glial fibrillary acid protein (GFAP)-positive cells and synaptophysin (SYNAPTO)-positive neuronal cells forming solid areas. (adapted from 52). **B.** Histopathology specimen (HE stain) of a RGNT (rosette-forming glioneuronal tumor; adapted from 53). **C.** PAS stain of a ganglioglioma displaying perivascular lymphocytic infiltrates and weakly PAS-positive eosinophilic granular bodies (x100 magnification; adapted from 54). **D.** Histopathology of Neurocytoma. Immunohistochemistry for NeuN showing neuronal differentiation of tumor cells adapted from 55).

exhibit WHO grade I behavior (Table 1). The Ki-67 labeling indices of most glioneuronal tumors are low, mostly 1–2% and generally less than 5%; however, in PGNT, 13–14% of total reported cases have shown increased proliferative indices (1, 34, 42) in line with high grade gliomas (56–58). The pathology of glioneuronal tumors is complex, requiring significant neuropathological expertise for interpretation. Notably, unlike most other glioneuronal tumors, GNTNI and WHO grade III variants of GGs, such as anaplastic ganglioglioma and atypical neurocytoma behave more similar to other high-grade gliomas, also carrying a poorer prognosis. DLGNT was introduced in the 2016 WHO classification of central nervous system tumors (1). A number of publications have since focused on this entity (1, 2, 35, 45–51, 59) (Figure 1). DLGNT mainly occurs in children and is mostly characterized by leptomeningeal growth, although Appay *et al*. described cases that are neither diffuse nor leptomeningeal, concluding that DLGNT may represent a "spectrum that has yet to be fully clarified" (45). DNET is a benign, glioneuronal neoplasm also part of the differential for other glial tumors including glioma, ganglioglioma, pilocytic astrocytoma or diffuse astrocytoma (4, 5). DIA/DIG is under-represented in the literature, with fewer than 20 cases, it is present generally in infants less than 24 months and displays prominent desmoplasia (60–64) (Figure 3).

Molecular characterization

While the underlying biology for the different glioneuronal tumor subtypes remains unclear (4), large scale genomic and epigenomic analyses have provided more insight into genetic alterations (4–9). Data is still emerging but the rarity of glioneuronal tumors means it may take some years to fully explore. Stone *et al*. suggest that most glioneuronal tumors fall within two major groups: group 1 containing a higher proportion of tumors with a ganglioglioma-like appearance

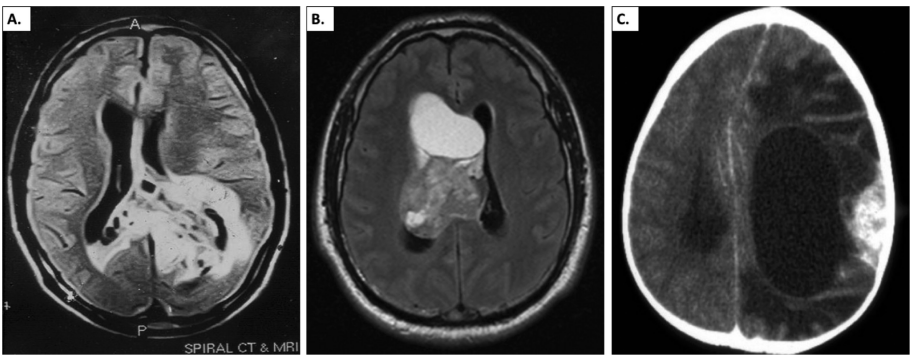

Figure 3. Radiographic appearance of glioneuronal tumors. A. Papillary glioneuronal tumor. Non-contrast magnetic resonance imaging showing hyper-intense lesion involving the left temporal and parieto-occipital regions. The tumor is crossing the midline to the right parietal region (adapted from 62). **B.** Axial FLAIR (fluid-attenuated inversion recovery) MR image showing a right ventricular mixed solid/cystic mass limited by the septum pellucidum and ventricular walls. With heterogeneous enhancement on post-gadolinium sequences. Provisional diagnosis of central neurocytoma, later confirmed on pathology (adapted from 63). **C.** Radiologic appearance of desmoplastic infantile ganglioglioma (adapted from 64).

displaying more BRAF-V600E mutations while group 2 tumors are more DNET-like in appearance and display more FGFR1 mutations (4). BRAF is an oncogene mutated in many malignancies and more recently described in one-third of GGs and 20–25% of DNET and DIA/DIG (4, 5, 65, 66). Some gliomas and glioneuronal tumors are characterized by a fusion between the BRAF gene and the locus KIAA1549 (45) (Table 1). The fusion causes a constitutional activation of the tyrosine kinase domain of BRAF and a permanent activation of the MAP kinase (MAPK) pathway (6,66). The detection of a BRAF rearrangement can help distinguish cancers with favorable prognosis such as glioneuronal tumors from those with poorer prognosis, such as diffuse gliomas, including diffuse astrocytomas and oligodendrogliomas. It also has therapeutic implications, as targeted therapies against mutated BRAF-V600 protein are being developed (vemurafenib, dabrafenib) (67). BRAF V600E mutations were identified by DNA sequencing in 33% of GGs and 27% of DNTs (8) and by immunohistochemistry in 29.5% of cases, 61.5% representing GG/GC/anaplastic ganglioglioma (5). Results can be discordant between immunohistochemistry and other molecular tests (5) illustrating the ongoing challenges in harnessing molecular testing for glioneuronal tumors and the as yet limited ability to draw conclusions with respect to diagnosis, prognosis and management. To date, the presence of BRAF mutation has not been associated with clinical presentation, imaging features or resolution of seizures postoperatively, acknowledging the limited data available. This is however evolving with recent descriptions of neuronal/glioneuronal tumors arising from the diencephalic region with a BRAF V600 mutation rate of 75% exhibiting clinically aggressive behavior (68). In pediatric GGs a worse recurrence-free survival of tumors with BRAF V600E mutation was reported by Dahiya *et al*. (69). Other identified mutations include PIK3CA (RGNTs and mixed RGNT/DNET) (69) with implications for targeting the PI3K/AKT/mTOR pathway (70,71), FGFR1 mutations (20, 71–76), H3K27M-mutations (77, 78), SLC44A1-PRKCA and NOTCH1-PRKCA fusion (79), IDH1 mutation (80) and 1p 19q alterations (78, 81) (Table 1).

Imaging Features

The features of glioneuronal tumors on diagnostic imaging are highly variable. Limited data exists, hampering an in-depth analysis of radiographic-pathological correlation. Hybrid features have been noted in many tumors. In general, radiological studies acknowledge overlap of imaging features between glioneuronal tumors and other tumors, which complicates radiographic diagnosis (82). Large tumors demonstrate cystic degeneration and necrosis, hemorrhage, contrast enhancement, and regions of low apparent diffusion coefficient consistent with patterns seen with other high-grade pediatric brain tumors (83). Broadly, glioneuronal tumors are characterized by the presence of a solid/cystic mass in periventricular location with septations and a solid inner component (84) (Figure 3). Attempts have been made to radiographically classify long-term epilepsy-associated tumors, of which glioneuronal tumors form a significant component. A number of small series have described imaging features of PGNT (42, 85), RGNT (40, 41, 86), DLGNT (45–47, 87), GG (56), neurocytomas (16), DNT (8). PGNT, RGNT and GNTNI were added to the WHO classification in 2007 hence imaging reviews are more recent and evolving (2). Most of these tumors are located in the supratentorial region (69%), however, spinal (23%) and

disseminated disease at primary diagnosis (8%) are also described (7). They exhibit variable contrast enhancement with GNTNIs appearing mostly as solid tumors in 73% of cases; about 19% appear as cystic with a mural nodule under T2-hyperintensity and T1-hypointensity (7) (Figure 3A (62)). The most common site of glioneuronal tumors is the temporal lobe followed by frontal lobe (8). Tumors with a high Ki-67 index (≥5) are more likely to exhibit perilesional edema and ring enhancement on magnetic resonance imaging (MRI) (10). DLGNT was added to the WHO classification in 2016 and publications have increased since (1, 2, 45–50, 55). In DLGNT, MRI is generally consistent with diffuse leptomeningeal enhancement predominantly and multiple cystic-solid lesions along the neural axis (49, 59, 87) but may present more atypically as well (45, 88). GGs appear cystic-solid or solid with long T1 and T2 signals with associated calcification (89). Often there is limited edema, and they may display no or mild contrast enhancement. Neurocytomas can be ventricular or extraventricular. Extraventricular neurocytomas are usually cortically based and infiltrative without peritumoral edema or intratumoral hemorrhage (Figure 3B (63)). DIA/DIG on computed tomography and MRI appear as large superficial large cerebral masses with solid and cystic areas (60). The solid component of the tumor frequently shows contrast enhancement (60) (Figure 3C (64)). Ultimately, the differential diagnosis of these findings includes low-grade glioneuronal tumors and low-grade gliomas.

MANAGEMENT, CLINICAL RESPONSE AND LATE EFFECTS

Generally, surgical resection is the corner stone of seizure management for patients with glioneuronal tumors. The purpose of resection in glioneuronal tumors is twofold: to alleviate symptoms (secondary to CSF flow disruption, seizures and/or increased intracranial pressure) and to achieve debulking in the context of more aggressive tumor subtypes (13, 26, 90).

Symptom control and surgical management

Glioneuronal tumors presenting with hydrocephalus (DLGNT, RGNT) should be considered for urgent ventriculoperitoneal shunting surgery which can result in complete symptom resolution (14, 25, 49). Surgical resection for debulking, and restoration of cerebrospinal fluid (CSF) flow when impeded, is the standard management (12, 25). Often the decision is made in multidisciplinary settings as these tumors are rare and the management is fraught with significant risks for late effects, particularly in patients with tumors that display more benign behavior who generally survive for longer. Several studies suggest that upwards of 70% of patients experience complete resolution of seizures post-operatively (8, 12, 90). Prognosis can be good with a progression-free survival (PFS) in 85 to 95% of patients (12). Gross total resection (GTR) is superior to subtotal resection (STR) and tumors with a lower Ki-67 index and a lower WHO grade have a better prognosis as compared to those with higher index and higher grade (7, 12). Only about 50% of patients undergo GTR prompting the administration of adjuvant radiotherapy and chemotherapy in some patients although due to limited evidence the benefit thereof remains unclear (18, 20, 40).

Systemic Management and Radiation Therapy

Radiation therapy (RT) is generally considered for recurrent and higher-grade tumors such as neurocytoma, anaplastic ganglioglioma and GNTNI. Acharya *et al*. analyzed 150 patients with unresectable pediatric low-grade gliomas and glioneuronal tumors to identify prognostic features in patients treated with RT using clinicopathologic and molecular data (18). RPA (Recursive Partitioning Analysis) yielded low- and high-risk groups with 10-year overall survival (OS) of 95.6% versus 76.4%. High-risk tumors included diffuse astrocytoma or location within thalamus/midbrain while low-risk tumors included pilocytic astrocytoma/ganglioglioma located outside of the thalamus/midbrain (18). The prognosis was independent of BRAF status but within the high-risk group, delayed RT (defined as RT after at least one line of chemotherapy), was associated with a further decrement in OS (18). The administration of chemotherapy is also heterogenous. Johnson *et al*. (16) carried out a comprehensive literature review of central neurocytoma regarding administration of chemotherapy. They identified 18 citations (39 cases of adult and pediatric central neurocytoma treated with chemotherapy) and found that nine patients with recurrent neurocytoma received temozolomide (TMZ) noting significant heterogeneity in chemotherapy administration (16). Chen *et al*. reported on long-term outcomes of 63 neurocytoma patients who received adjuvant radiotherapy after surgical resection (19). With a median follow-up of 69 months the 5-year OS and 5-year PFS were 94.4% and 95% after GTR + RT, 96.4% and 100% after STR + RT, and 100% and 90.9% after PR + RT (19). RT after incomplete resection led to OS and PFS comparable to those for GTR with excellent outcomes and limited late toxicity suggesting that adjuvant RT is a reasonable option for neurocytoma patients with incomplete resection. Radiosurgery as an alternative has also been proposed as an option with 5- and 10-year local tumor control rates 93% and 87%, respectively, and the 5- and 10-year PFS rates 89% and 80%, respectively (91, 92). The use of chemotherapy, targeted agents, and Bevacizumab is only subject of case reports and small studies and benefit remains unclear (93–97). For disseminated, recurrent and high-grade disease, definitive radiotherapy or radiochemotherapy is considered and treatment may overall reflect that of other high-grade gliomas due to the histologic and natural history similarities they share (7, 17). Generally, the outcome in patients with glioneuronal tumors can be broadly discussed in the context of progression of disease, transformation to higher grade tumors and the long-term complications of treatment or late effects. In a series of patients treated at the St. Jude Children's Research Hospital (1986 to 2015), progression of disease and transformation to higher grade glioma accounted for 66% of the mortality (24). Other causes included secondary malignancy, shunt infection/sepsis, suicide and motor vehicle accidents (24). In this series, the median age at death for the cohort was 14.26 years (range, 0.58–32 years), and the median time to death from diagnosis was 4.02 years (range, 0.21–24 years). Overall, our understanding of the optimal management and the outcomes of glioneuronal tumors remains limited due to the rarity of these tumors and the data originating from small series and literature reviews. With ongoing molecular characterization and the paralleled progress of targeted agents, data continues to evolve.

Late effects

Despite more favorable survival outcomes as compared to other more aggressive gliomas, significant late effects are associated with glioneuronal tumors. Late effects are likely multifactorial, stemming from a combination of tumor presence, surgical resection, and adjuvant management including systemic agents and RT. The lack of data surrounding optimal management and outcomes also extends to lack of clarity surrounding the burden of late effects on patients with glioneuronal tumors. Ehrstedt *et al*. carried out a cross-sectional long-term follow-up evaluation on 28 children and adolescents (0–17.99 years), with a mean follow-up period of 12.1 years (23). They identified postoperative gain in cognitive function in seizure-free patients, but at a relatively low level, and high levels of anxiety and depression (23). In a series of 51 patients with low grade glioma and glioneuronal tumors managed at the St. Jude's Children's Research Hospital, USA, (1986 to 2015) with a mean age at diagnosis of 6.47 months (range, 0.17–11.76) and mean duration of follow-up of 11.8 years, 96% of patients experienced at least one long-term deficit, such as endocrinopathy and obesity (51%), neurological deficit and seizure (43%), visual and hearing loss (56%), neurocognitive impairment (49%), cerebrovascular disease and scoliosis (27%) and secondary malignancy (14%) (24). Late effects correlated with tumor location (hypothalamic/optic pathway), administration of radiation therapy and more chemotherapy regimens (24). According to Upadhyaya *et al*., early psychological intervention should be included as part of the multidisciplinary management approach of children with both glioneuronal tumors and low-grade gliomas to reduce the risk of suicide in vulnerable subjects.

CONCLUSION

Glioneuronal tumors are uncommon tumors comprised of glial and neuronal components. They generally display indolent behavior but can behave aggressively. They are pathologically and radiographically complex, and classification hinges on advancements with respect to molecular analysis to allow for future personalized treatment which may improve outcomes. Currently, cases are best managed in multidisciplinary settings with the role of adjuvant treatment in the form of chemotherapy and radiation therapy beyond surgery remaining unclear. In depth counselling regarding late effects is paramount due to the burden of long-term life altering sequelae in long-term survivors. The creation of robust registries and tumor sequencing is imperative to allow for improvement of outcomes in the long term.

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Gliomas

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