Insights from translational research studies in malignant brain tumors are paving the road toward personalized treatments

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Interests:
Malignant glioma
Metastatic brain tumors
Metabolism and imaging of brain tumors
Mouse modeling of gliomas and brain metastases

Purpose and Overview:
The goal of this presentation is to review the state of the field of malignant gliomas including diagnosis and management of low and high grade gliomas. In addition, barriers to progress will be discussed and multiple projects involving translational research approaches to developing effective personalized therapies for brain tumor patients will be presented.

Objectives:

1. To differentiate the grades of glioma by histopathological features, imaging characteristics and response to treatment.

2. To identify the major barriers to progress in the development of novel therapies for gliomas and demonstrate the importance of developing accurate preclinical models of the disease that can be used in an integrated preclinical testing platform.

3. To discuss the role of 2HG imaging in the diagnosis, prognosis and treatment response measurement in IDH-mutated gliomas.

Part 1: General Background

Overview:

The Central Brain Tumor Registry of the United States (CBTRUS) estimated 51,410 new cases of malignant and non-malignant primary brain tumors in 2007. Among children, the incidence is 4.5 cases per 100,000 person-years. Among adults, malignant tumors are estimated at 7.3 per 100,000 person-years and non-malignant tumors estimated at 9.2 cases per 100,000 person-years. An early peak in incidence starts at birth and extends to 4 years of age; after age 24, a gradual rise in incidence occurs, leading to a second peak at 50-79 years. For 2008, the SEER Cancer Statistics Review estimates that cancer of the brain and nervous system will account for 1.5% of all new cancer cases and 2.3% of cancer deaths annually (SEER 1975-2005). The relative risks of CNS malignancy is 1.38 male to female, 3.18 elderly to young adult and 1.86 Caucasian to African-American. In children, CNS tumors are the most common solid neoplasms and are the second leading cause of cancer death in patients younger than 15 years of age (SEER 1975-2005).

Gliomas account for 36% of all primary brain tumors and 81% of malignant tumors (CBTRUS) Among these, glioblastoma is the most common, accounting for at least 50% of cases. Meningiomas account for 32.1% and pituitary tumors represent between 5% and 15% of all brain tumors. Nerve sheath tumors, lymphoma, medulloblastoma, and craniopharyngioma range from 0.8% - 9%. Spinal cord neoplasms account for fewer than 15% of CNS tumors and 10% of these represent spinal metastases from a primary intracranial tumor. Of all primary tumors of the spinal cord, schwannomas and meningiomas each account for 30%, ependymomas 13%, sarcomas 12%, astrocytomas 7%, and chrodomas 4%. The distribution of CNS tumors varies with age: 90% of adult brain tumors are supratentorial, whereas 70% of childhood brain tumors arise in the posterior fossa.

The biological potential of CNS neoplasm depends largely on three factors: (1) the histology and degree of malignancy (grade) of the tumor; (2) the anatomic compartments involved (cerebral hemisphere, basal ganglia, posterior fossa, brainstem, third ventricle, visual system, spinal cord, etc.); and (3) the spatial delimitation of the tumor (e.g. diffuse, circumscribed, multifocal)(Maher and McKee In Press). CNS tumors of low histologic grade may have as poor a prognosis as high-grade malignancies if they are considered surgically unresectable either because they exhibit a diffusely infiltrating growth pattern, they involve a critical anatomic structure or that they are technically unapproachable by surgery.

Metastatic brain tumors, operationally defined as the growth of a tumor in the brain that had its origins in a cancer derived from an organ outside the central nervous system, is estimated at approximately 200,000 cases per year. Current treatment revolves around the number of metastatic lesions and whether radiosurgery or whole brain radiation is appropriate given the number and location of the tumors as well as the performance status of the patient (Maher, Mietz et al. 2009). The discussion at Medical Grand Rounds will focus on the malignant gliomas (below) but the translational studies described for the gliomas also apply to brain metastases.

Malignant Gliomas

Approximately 65% of malignant brain tumors are gliomas, representing tumors derived from astrocytes and oligodendrocytes or possibly from neural stem cells and early glial progenitors. While the precise cell of origin remains unclear, tumor subtypes are named for the normal glial cell that they most closely resemble histologically. Tumor grade, established by the

World Health Organization, reflects characteristic features related to degree of anaplasia, presence of mitotic figures, and the presence of microvascular proliferation and/or necrosis (Louis, Ohgaki et al. 2007). Overall survival is tightly correlated with tumor grade, with patients of low grade astrocytomas (WHO grade II) having a 10-12 year median survival, anaplastic astrocytomas (WHO grade III) having a 3-5 year median survival and GBM (WHO grade IV) having a median survival of approximately 15 months. Oligodendrogliomas account for approximately 10% of gliomas, and approximately 50% of these tumors are well recognized for their sensitivity to alkylating agents and to radiation therapy. Graded either low grade (II) or anaplastic (III), the chemosensitive subset are characterized by chromosomal deletions of 1p and 19q. To date, no clear gene candidates have emerged from these regions that account for the response to chemotherapy. A small subset of gliomas are of mixed cellular phenotype and thus are named for the cells they resemble. Oligoastrocytomas demonstrate histologic features of both oligodendrogliomas and astrocytomas and gliosarcomas are tumors that have elements of both sarcoma and astrocytoma (Louis, Ohgaki et al. 2007). The cell of origin for these mixed lineage tumors is unknown although they likely derive from an early glial progenitor cell that retains the capability of differentiating into both lineages. The characteristic clinical feature of all glioma grades is diffuse infiltration throughout the brain. There is a clear progression from low-grade to high-grade astrocytoma with a dramatic increase in mitotic rate and induction of angiogenesis. Although a hallmark feature of the gliomas is their ability to widely invade normal brain parenchyma, except in rare cases, they do not metastasize outside the central nervous system.

Glioblastoma (WHO IV)

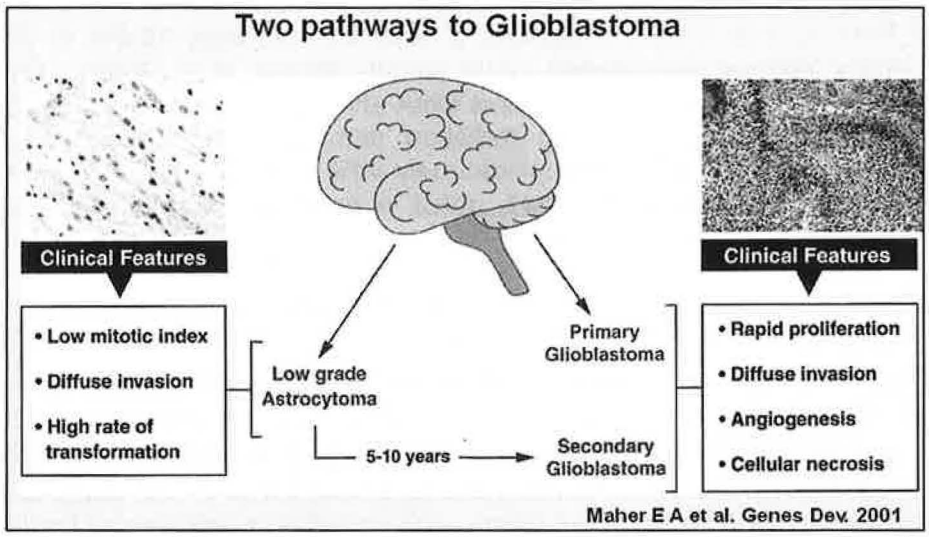
Glioblastoma (GBM) is a disease that strikes without warning, often manifested by a single seizure or a few days of progressive headaches, yet workup reveals a large mass that was likely present for months. Its stealth-like behavior belies the hallmark feature of this deadly cancer; that it infiltrates normal brain diffusely, early in the disease course and over long distances, making it impossible to detect early or to cure surgically. Survival times are short. With maximal therapy, the median survival is 14.6 months and the 2 year survival is 26% (Stupp, Mason et al. 2005). The neurological impact of the tumor is determined by its location in the brain – left temporal lobe masses leading to speech and language problems, frontal lobe tumors leading to weakness and eventually paralysis, and corpus callosum masses (the classic presentation) leading to confusion. Superimposed are treatment associated changes in neurocognition which impair daily functioning and work performance. The social and economic toll on patients and families is magnified by the age distribution of the disease with ~75% of patients being between the ages of 35 and 60, working men and women who often have young families. The need for novel therapies to impact survival is clear. The potential impact of effective therapies on neurological function and quality of life is enormous. In some cases with response to treatment, dramatic neurological recovery has been seen, with resolution of abnormal signal on MRI and restoration of normal speech, motor function and cognition associated with tumor cell kill. This is a major distinguishing feature of malignant gliomas as contrasted with the neurodegenerative diseases and stroke, for which neurological impairment is permanent due to neuronal cell death. In glioblastoma, infiltrating tumor cells disrupt the fidelity of electrochemical communication within synaptic networks without irreversible destruction of neighboring neurons or axons, until late in the disease when the increasing tumor mass and profuse angiogenesis that is highly prone to leaking, thrombosis and necrosis, exerts overwhelming pressure and irreversible damage. Thus, early intervention with an effective treatment has the potential not only for improving survival but also for restoring neurological function completely. The stakes are high, however, since survival times are short and the window of opportunity to make an important impact closes early in the disease course.

Anaplastic astrocytoma (WHO grade III)

Anaplastic astrocytomas may arise *de novo* or develop from low-grade lesions. They are characterized histologically by nuclear atypia, increased cellularity and a significant increase in mitotic rate over that seen in low-grade lesions without induction of neovascularization. MRI demonstrates enhancement of tumor following administration of gadolinium in approximately 80% of cases. The median age at diagnosis is 41 years, patients presenting with symptoms similar to those describe above for patients with GBM. Survival is significantly longer than glioblastoma, ranging from 3-5 years. Often linked with GBM and referred to as "high grade gliomas", it must be clarified that the anaplastic astrocytomas (grade III) have a much better prognosis than grade IV and, with concurrent radiation and temozolomide (see below), appear to have prolonged remissions.

Low grade gliomas (WHO II)

For the purposes of simplicity in nomenclature, we refer to the grade II tumors by their common name, low grade gliomas. While a great deal of attention both in terms of both basic and clinical research is focused on glioblastoma (GBM), relatively little is known about the biology of low grade gliomas, which are also uniformly fatal, albeit with a median survival of 10-12 years. The tumor cells are well differentiated, exhibit robust glial marker immunoreactivity and are not associated with neovascularization or cellular necrosis. MRI often demonstrates a diffuse large mass that is hypointense on T1-weighted imaging and does not enhance following administration of gadolinium. Low grade gliomas are more common in younger patients, with a

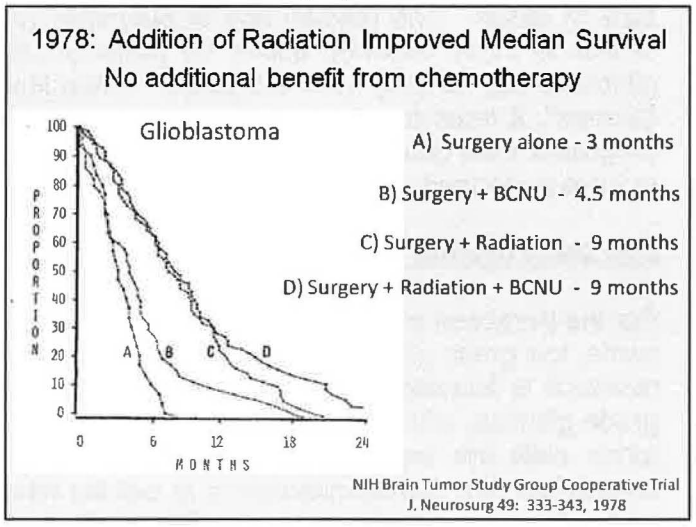


median age of 30, and a greater than 80% risk of tumor transformation to GBM. These 'secondary GBMs' are histologically and clinically indistinguishable from primary GBM and carry the same poor prognosis. Current treatment of low grade gliomas is largely ineffective, with radiation being the only modality which has been shown to prolong progression-free survival although without an impact on overall survival or the rate of transformation to GBM. Thus, currently, patients undergo surgical debulking and then are simply followed with serial MRI scans, 'waiting' with great fear for the deadly progression to GBM and the associated short survival time. For those patients who do not progress to GBM, they die of widely infiltrating tumor. The lack of therapeutic options for low grade gliomas stems from the limited understanding of basic biological mechanisms underlying both the slowly proliferative state as well as the trigger to GBM. The importance of focusing on this deadly cancer derives both from

the young patients who have no treatment options and from the potentially important information that can be gleaned from studying the transition to GBM.

Treatment of Glioblastoma: Historical through 2012

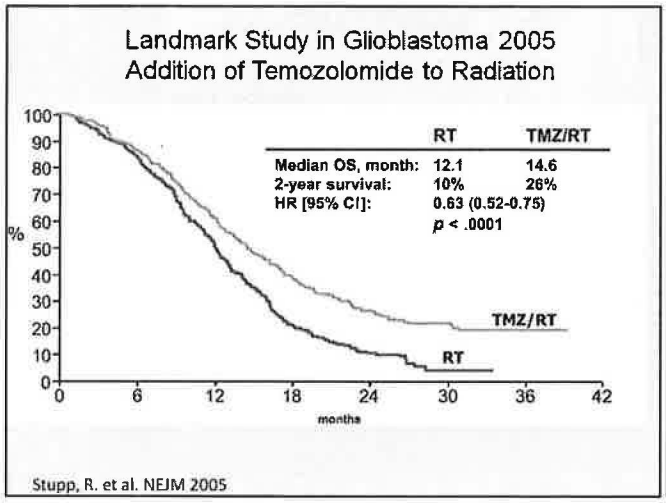
In 1978, it was established that external beam radiation improved median survival from 3-9 months (Walker, Alexander et al. 1978). In the same study, it was clear that addition of chemotherapy, carmustine (BCNU) added no benefit after surgery alone or after surgery followed by radiation. Over the subsequent 15 years, many drugs were tried in the adjuvant setting with no improvement in overall or progression free survival was seen. The drugs tested were those that were having benefit in other cancers, without regard to whether the drug was able to penetrate the blood brain barrier. Various radiation schedules, field sizes and doses were evaluated and none was able to improve over the ~9 months survival.

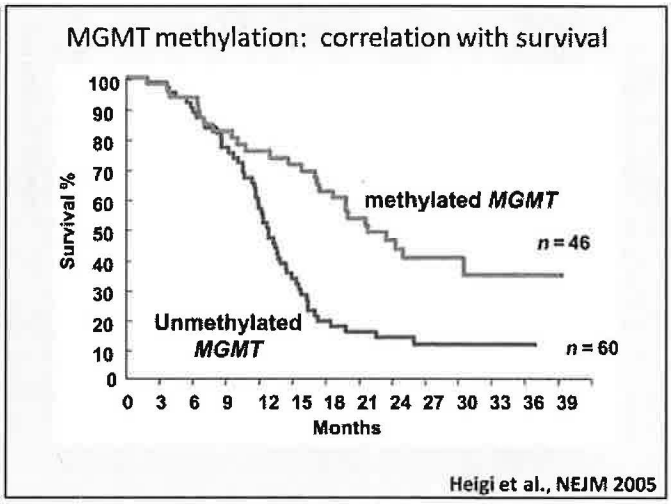


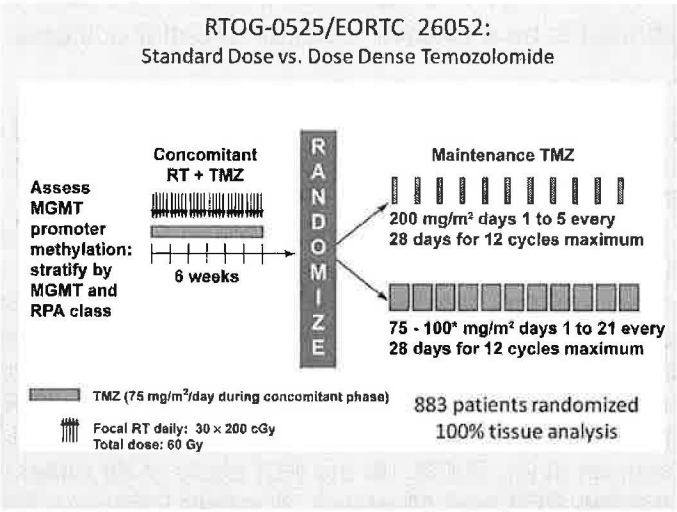
The treatment of glioblastoma has evolved over the past several years with the demonstration that treatment with temozolomide, an oral alkylating agent, when given concurrently with radiation therapy as initial therapy after surgical resection or debulking, and as adjuvant therapy for six cycles, improved overall survival from 12.1 to 14.6 months and 2 year survival from 10.4% to 26.5% when compared to surgery followed by radiation alone (Stupp, Mason et al. 2005). Correlation of methylation status of MGMT, a gene that repairs DNA after alkylation, with survival in patients treated with combined temozolomide and radiation demonstrated marked prolongation of survival in the patients with MGMT, with approximately 40% alive at 3 years (Hegi, Diserens et al. 2005). The predictive potential of MGMT status is currently under evaluation in a large multicenter international study (RTOG 0525).

In the mid-1990's, clinical trials with temozolomide were initiated. Temozolomide is a second generation alkylating agent, is 100% bioavailable, has 100% blood brain barrier permeability and its clearance is unaffected by coadministration of antiepileptic therapy, antiemetics or decadron. The side effect profile was excellent without significant nausea/vomiting, and myelosuppression only requiring dose reduction in 3% of patients (Newlands, Stevens et al. 1997). An international phase 2 trial of 162 patients with recurrent anaplastic astrocytoma or anaplastic mixed glioma was undertaken with temozolomide being given at a dose of 150-200 mg/m^2 daily for 5 days, every 28 days. Objective response rate was 35%, with 8% complete response and 27% partial response and stable disease pf 26%. The six month progression free survival (PFS) was 46%, longer than any drug that had ever been used for glioma. With the results and the favorable side effect profile, this study provided the basis for FDA approval of temozolozmide initially for anaplastic gliomas (Yung, Prados et al. 1999). An international randomized phase 2 trial was conducted which compared temozolomide with procarbazine in 225 patients with GBM at first relapse. The six month PFS was 21% (temozolomide) vs. 8% (procarbazine), $p<0.008$, and median PFS 2.89 months vs. 1.88 months, respectively ($p<0.0063$) but no difference in 6 month or median overall survival (Yung, Albright et al. 2000). Alternative schedules were tried in the recurrent and upfront settings for GBM and an

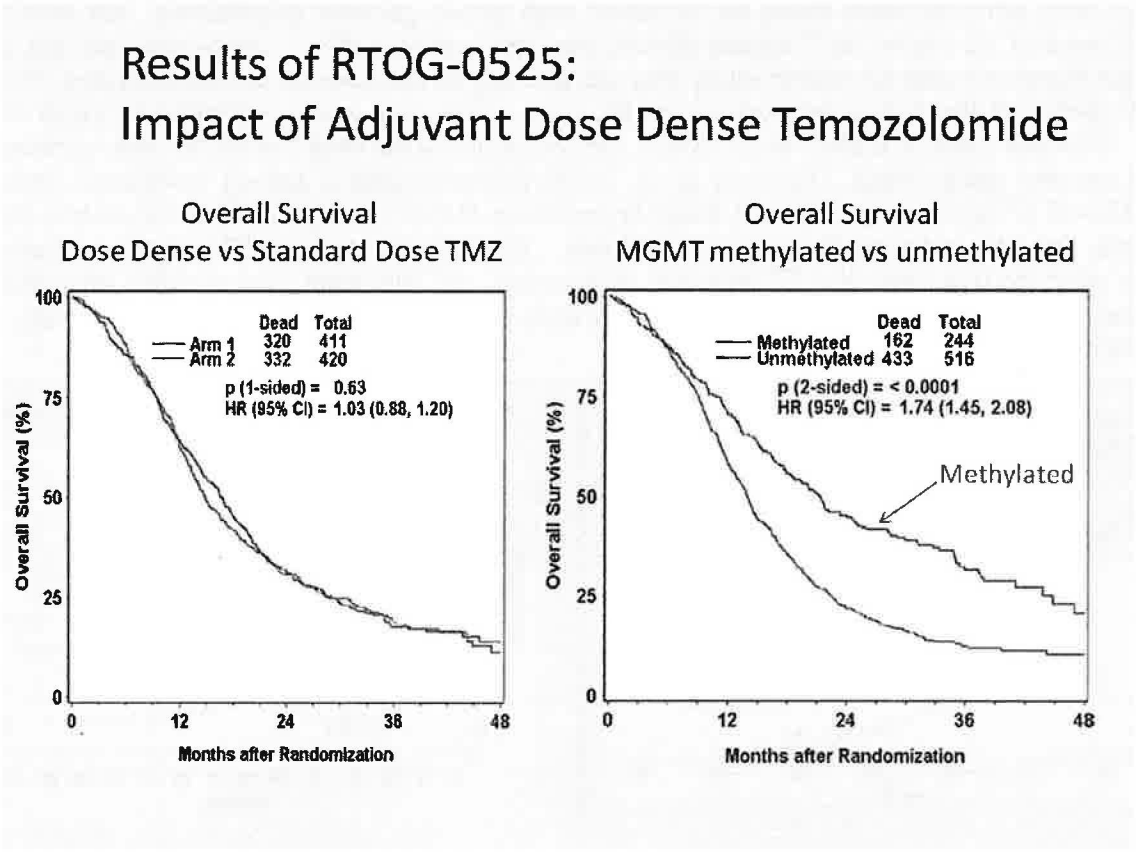
extended daily administration study for recurrent high grade gliomas established the maximally tolerated dose of 75 mg/m^2 for 7 weeks (Brock, Newlands et al. 1998). These data set the stage for a large European and Canadian study that added daily temozolomide to radiation and followed it with 6 cycles of the 5 day regimen every 28 days. The data were published in 2005 in two studies. The first (Stupp, Mason et al. 2005), demonstrated a survival benefit for the combination and the second study (Hegi, Diserens et al. 2005) demonstrated a strong correlation between methylation of O^6-Methylguanine-DNA methyltransferase (MGMT) and overall survival in a subset of patients for whom tumor tissue was available. MGMT encodes a DNA-repair protein that removes alkyl groups from the O^6 position of guanine, an important site of DNA alkylation by temozolomide. Methylation of MGMT leads to loss of function of the repair mechanism after treatment with temozolomide.







After identification of the MGMT correlation, several in vitro studies were done to determine the temporal course of depletion and repletion of the MGMT when exposed to temozolomide, with the idea that prolonging the exposure to MGMT in a non-methylated tumor cell would effectively deplete the supplies and turn a "non-responder" into a "responder" and lead to longer survival. On the basis of these data, a large international randomized study was undertaken to determine if giving adjuvant temodar 21 out of 28 days would improve survival over the 5 out of 28 day standard cycles. Eight hundred and eighty three newly diagnosed glioblastoma patients were enrolled prior to radiation and were treated with the standard protocol schedule (Temodar 75 mg/m2/day throughout radiation). Patients were then randomized at 4 weeks post radiation to either standard dose (150-200 mg/m2/day x 5/28 days x 6-12 cycles) or the dose dense arm (75-100 mg/m2/day x 21/28 days x 6-12 cycles). Tissue was collected from every patient in order to determine if the MGMT methylation status was predictive of response. The results of the study (RTOG-0525) were presented at ASCO in 2011 (M.Gilbert, PI).



There was no change in overall survival and no change in progression free survival (data not shown). MGMT methylation status was confirmed to be a positive predictor of better outcome.

Antiangiogenic therapy:

Bevacizumab approved for the treatment of recurrent GBM

The use of specific antiangiogenic agents for the treatment of malignant gliomas is an area of intense clinical investigation. Bevacizumab was FDA approved for the treatment of patients with progressive glioblastoma after upfront treatment with temozolomide-based therapy. Approval was based on 2 prospective phase II studies, BRAIN and NCI 06-C-0064E which were done in recurrent glioblastoma patients and response was measured radiographically in the setting of stable or decreasing steroid dose. In the BRAIN study, ORR was 28% (24 of 85 patients), median duration of response was 5.6 months, 6 month-PFS was 42.6% and median OS was 9.2 months (Friedman et al., 2009). In the NCI study of 48 patients. using the same criteria, ORR was 19.6%, median PFS was 16 weeks, 6 month PFS was 29% and median OS was 31 weeks.

The toxicity profile of bevacizumab in recurrent high grade gliomas (glioblastoma and Anaplastic astrocytoma) are similar to the toxicities reported for other cancers and include low-grade bleeding, hypertension, impaired wound healing, and proteinuria. However, the rate of other serious adverse events, such as gastrointestinal perforation, reversible posterior leukoencephalopathy syndrome and wound-healing complications are low in glioblastoma (Chamberlain et al., 2010). Importantly, the rate of life-threatening intracranial hemorrhage is rare.

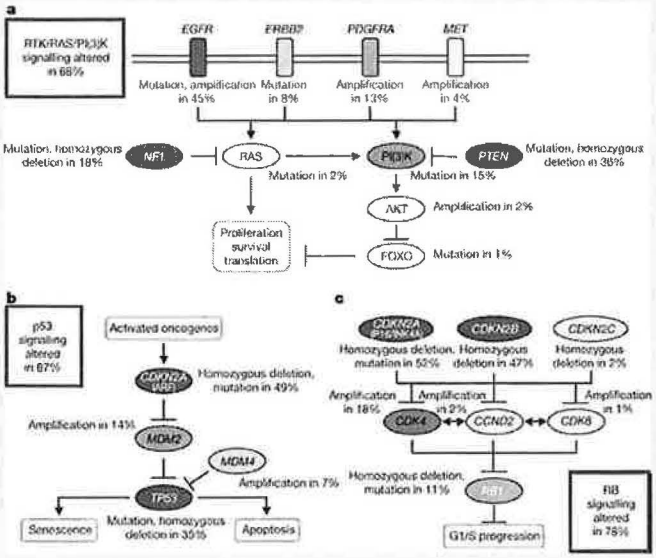
Bevacizumab combination studies for recurrent disease:

Several small studies have established the activity of bevacizumab in combination with irinotecan or other agents. There is considerable debate as to whether the addition of chemotherapy improves response rates or overall survival. No direct comparison studies have been done. However, In the BRAIN study described above, a second arm was done concurrently although the study was designed to be a noncomparative trial (Friedman et al., 2009). In the bevacizumab + irinotecan arm, 85 patients were enrolled. There was an ORR of 37.8%, 6-month PFS of 50.3%, and median OS of 8.7 months. Of the patients treated with bevacizumab alone or bevacizumab plus irinotecan, 46.4% and 65.8%, respectively, experienced grade >/= 3 adverse events, the most common of which were hypertension (8.3%) and convulsion (6.0%) in the bevacizumab-alone group and convulsion (13.9%), neutropenia (8.9%), and fatigue (8.9%) in the bevacizumab-plus-irinotecan group. Intracranial hemorrhage was noted in two patients (2.4%) in the bevacizumab-alone group (grade 1) and in three patients (3.8%) in the bevacizumab-plus-irinotecan group (grades 1, 2, and 4, respectively) (Friedman et al., 2009).

<u>Molecularly targeted therapies: Poor track record in GBM</u>

For decades, signature mutations in GBM and the lower grade gliomas have been well known. There are striking differences between low grade gliomas and GBM, beyond proliferation. The complex GBM genome is characterized by the classic cancer pathways, including activation of the RAS-MAPK pathway, most commonly driven by EGFR amplification, and dysregulation of the PI3Kinase pathway, due frequently to deletion of PTEN. In contrast, the low grade genome is significantly less complex with fewer than 1/3 as many copy number alterations and only p53 mutations are found among the cancer-relevant changes (Fults, Brockmeyer et al. 1992). The only known oncogene is PDGFRα, which is amplified in approximately 15% (ref) with gene overexpression in over 50% of tumors. Most recently, identification of mutations in isocitrate dehydrogenase (IDH) have been described in excess of 70% of grade II and III gliomas, and the secondary GBMs that evolve from them (Dang, White et al. 2009). Transition to secondary GBM is heralded by a dramatic increase in the number of copy number changes, yet only 30% are held in common with primary GBM (Maher, Brennan et al. 2006) suggesting that progression to secondary GBM occurs along a different molecular

Summary of The Cancer Genome Atlas Project in GBM



TCGA Research Network *Nature* 2008

pathogengenetic route than primary GBM. Of the overlapping genes, most are common cancer genes, including p16, p19, PTEN, RB, p53 and PDGFRα.

Publication of the GBM analysis in The Cancer Genome Atlas project (The Cancer Genome Atlas Research Network, 2008) (2008)has provided a detailed view of the molecular aberrations in this deadly cancer. Two hundred and six GBMs were studied and the most striking finding was the predominance of alterations in 3 signaling pathways. RTK/RAS/PI3K was altered in 88%, p53 was altered in 87%, and RB was altered in 78% of the samples, confirming much of what was already known. To date, an additional 300 tumors have been studied, increasing the numbers but not providing additional information.

Disappointingly, hundreds of small phase II studies of single and multi-agent regimens targeting the major signaling pathways outlined above (RAS/MAPK/PI2K, RB) in combination with cytotoxic and anti-angiogenic agents have been performed. To date, no drug or regimen has emerged with activity leading to a change in current treatment strategy described above.

Part 2: Translational studies in glioma: Trying to turn the tide

1) Preclinical Evaluation of a new drug or combination

A major point of failure in the development of new drugs for glioma is at the preclinical stage. The lack of good, faithful, predictive mouse models has been the subject of many meetings in the field and there is a commitment to improving this area significantly, with the expected benefit of improving the success rate for new drugs.

Genetically engineered glioma model systems

The development of model systems capable of validating critical targets (i.e., tumor maintenance targets), providing predictive information on the optimal genotypic context in which the target is rate-limiting (i.e., right patient subpopulation for a drug), and affording opportunities to test standard and novel drug combinations would transform the cancer drug development process for GBM. To date, a number of mouse glioma models have been developed which capture some phenotypic aspects of the human disease. Some of the most notable of these model systems include (RAD001) the Parada model harboring conditional NF-1 and p53 mutations that produce varying grades of disease from low-grade astrocytoma to highly penetrant malignant astrocytomas displaying characteristics of GBM (Zhu, Guignard et al. 2005); (2) the van Dyke model, which utilizes the inducible GFAP-CreER-T2 strain that, together with LSL-Kras T121 and conditional PTEN, produces Grade III/IV disease within 2 to 4 months (Xiao, Yin et al. 2005); (3) the Holland model, using the Nestin- and GFAP-directed RCAS system to produce a highly penetrant malignant glioma phenotype upon avian retroviral delivery of mutant EGFR in the setting of Ink4a/Arf deficiency or with combined expression of activated Kras and Akt (Holland, Hively et al. 1998); (4) the Bachoo 'stem transgenesis' model in which the VIII mutant of EGFR (EGFR*) transduction in Ink4a/Arf-/- NSCs or astrocytes produced Grade III astrocytomas (Bachoo, Maher et al. 2002) and (5) the Bachoo models using various mutations driven from a panel of new astrocyte-specific promoters.

Development of a Novel Human Orthotopic GBM Mouse Model:

For the past three decades, preclinical studies in GBM have relied on glioma cell lines cultured *in vitro* and injected into the subcutaneous space and, less often, intracranially, of immunocompromised mice for *in vivo* experimentation. Though widely used and useful for *in vivo* drug testing studies, these models have consistently been poor predictors of clinical response. Recent evidence has shown that the process of establishing stable cultures can lead to irreversible genomic changes both at DNA and RNA levels as well as on downstream

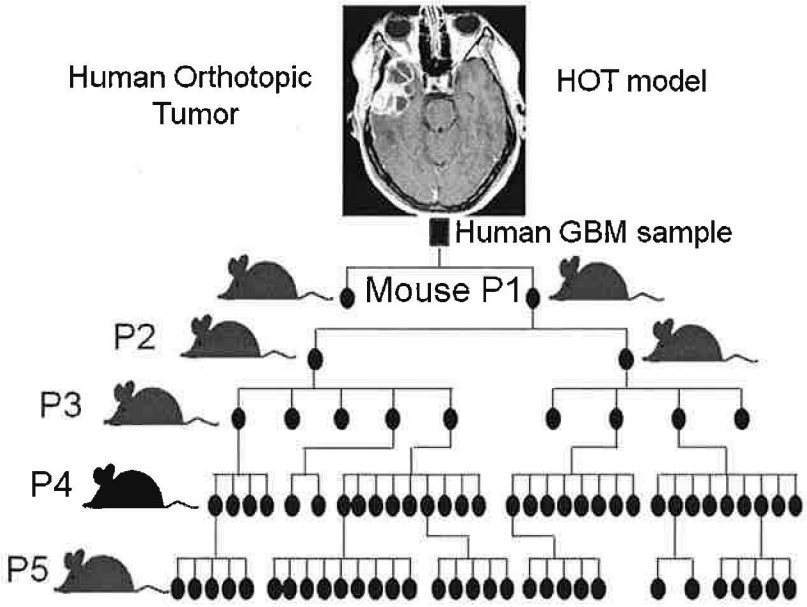
signaling pathways (Li, Walling et al. 2008). Subsequently growing cells lines as subcutaneous or intracranial tumors that have been adapted for cell culture fails to restore the constellation of mutations that are seen in the original tumor phenotype. This is clearly illustrated by the example of EGFR amplification, a signature mutation of primary glioblastoma, which is rapidly and irreversibly lost when cells are maintained under normal culture conditions (Li, Walling et al. 2008)

The translational focus of the neuro-oncology group at UTSW is to bring important questions from the clinical management of patients back to basic investigative team in the laboratory. The human orthotopic mouse model, led by Dr. Robert Bachoo, grew out of this effort, with a desire to generate tumor lines in mice that harbored the constellation of genetic changes that exist in the human tumors in order to recreate an accurate molecular and microenvironmental milieu in which to study tumor resistance. we have generated and characterized a series of primary GBM tumors obtained directly from patients at the time of initial surgical resection and propagated them orthotopically in SCID mouse brain. The feasibility and reproducibility of this approach has now been thoroughly examined in a total of 40 GBM tumors (37 primary and 3 secondary GBM) as well as 12 metastatic tumors (NSCLC, breast, melanoma, renal cell, colon cancer). We have shown that tumor lines maintained exclusively as orthotopic tumors by serially passaging in SCID mouse brain preserves all pathognomonic features of glioblastoma, including brisk proliferation (MIB>20-30%), nuclear atypia, diffuse single cell infiltration, and are immunoreactive for the classic astrocytic marker, GFAP (Marian, Cho et al. 2010). Most significantly, the critical feature of this model that makes it ideally suited for the studies being proposed here is the demonstration that the molecular profile of an individual patient's tumor cells are maintained over an indefinite period by multiple serial passages in mice. This was a surprising finding, given the assumption that all high grade solid tumors are inherently genetically unstable, as evidenced by genetic drift and clonal selection of cancer cells placed in hostile environments or exposed to conventional chemotherapeutic drugs. The stability of the glioblastoma genome in our model suggests that the NOD-SCID mouse brain provides an ideal permissive microenvironment that supports growth of the 'same' tumor cell clone present in the patient's brain, without significant modification or subselection of new tumor cell clones which are not representative of the original clinical tumor. The preservation of the molecular profile strongly supports the use of this model for our metabolic studies that rely on maintaining the 'in situ' genome in order to have a reliable 'functional readout' in the metabolic phenotype.

Sample procurement and intracranial injection: Under an IRB approved protocol at UTSW for the use of brain tumor tissue in basic research studies, freshly resected GBM (or brain metastasis) tumor is taken from the operating room directly to the Translational Neuro-Oncology Research Laboratory on ND3. In preparation for injection, cells are enzymatically dissociated and washed in normal saline. On average, each tumor yields approximately 200,000-300,000 viable cells and 50,000 cells are injected per mouse into the right caudate region of the SCID mouse brain, within 120 minutes of tumor resection.

Clinical and radiographic follow up of the orthotopic mice: Mice are examined twice weekly for signs of weight loss, loss of grooming, seizures, and focal motor deficits. Any of these signs is consistent with the presence of a tumor. Remarkably, each individual tumor line produced focal neurological deficits consistent with an expanding intracranial mass with similar latencies post-implantation. Brain imaging is performed routinely to document the presence and growth of an expanding mass by 1) 4.9T MRI and 2) by micro-PET. All mice were sacrificed when they became moribund, overall survival data between successive generations (Kaplan-Meier Analysis) shows no statistical difference ($p>0.5$) for each of the 6 primary tumors examined to date (data not shown). At time of sacrifice, the intracranial tumor mass (~100-

150mg/per mouse) is composed of ~ 5 x 10^8 live cells, which represents approximately a 5000 fold increase in tumor cell mass and sufficient tumor cell numbers to potentially establish over 100 orthotopic NOD-SCID mice with identical tumor cell genetics. This ability to expand the primary tumor mass while faithfully preserving the tumor phenotype is critical for several in vivo metabolic studies being proposed here, especially those proposing a kinetic analysis of labeled glucose which will require large numbers of mice with genetically identical tumors (see below).



Using the mouse in the clinical evaluation of a new drug. Recently, the mouse was used in combination with the neoadjuvant evaluation of Crenolanib, a potent and highly selective PDGFR inhibitor. Data will be presented showing how this mouse model can be used in the preclinical setting to work up a new drug before launching a full scale clinical trial. The key questions that, surprisingly, have not been asked in most drugs used in glioma clinical trials is whether the drug gets into the brain and tumor. The penetration may be different in GBM, which has a disrupted blood tumor barrier, than the low grade gliomas which have an intact blood brain and blood tumor barrier. This information is critical in the decision making for using the drug, yet very rarely done.

2) Cellular Metabolism: An examination of the brain tumor metabolic phenotype

A critical review of current treatments and of ongoing clinical investigation reveal that, despite recent success in a small percentage of patients, the majority of patients with brain tumors continue to face little over a year of survival that is marked by progressive physical and cognitive decline that culminates in death. As described above, treatment with cytotoxic agents, molecular targeted therapies, and anti-angiogenic therapies ultimately fail to kill or turn off the proliferating cell. In view of the complexity of the glioblastoma genome and the redundancy in the biochemical pathways altered as a result of the disrupted genome, mono- or even multi-agent targeted therapy may be only transiently effective. It has been clearly shown in other cancers (e.g. non-small cell lung cancer, chronic myelogenous leukemia, breast cancer, melanoma) that even when effective drugs can be targeted to specific mutations, the clinical benefits can be transient, with new mutations emerging that restore the tumor's proliferative capability. A complimentary approach to searching ever more deeply for the genes that drive tumor growth is to look downstream of the genetic changes and identify the steps in cellular

metabolism that 'fuel' proliferation. In this regard, tumor cell metabolism, modulated by activation of oncogenes and loss of tumor suppressor genes, has recently garnered much interest as harboring potential therapeutic targets.

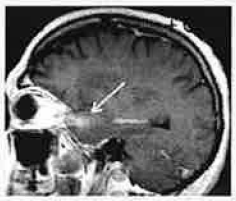
Progress in understanding cellular metabolism in the proliferating cancer cell has been significant over the past several years and novel mechanisms for metabolic control are emerging. The identification of mutations in metabolic regulatory genes, most recently in isocitrate dehydrogenase 1 (IDH1) in gliomas (Yan, Parsons et al. 2009), as well as the identification of enzyme isoforms that influence the tumor metabolic phenotype, strongly suggest that direct modulation of metabolic pathways is important for supporting tumor cell growth and ensuring survival, especially in an hypoxic tumor microenvironment. Beyond the demonstration that tumor cells rapidly use glucose and convert the majority of it to lactate (Warburg effect), the biosynthetic activities required to sustain rapid proliferation involve production of ribose-5-phosphate for nucleotide biosynthesis and production of fatty acids for lipid biosynthesis. Regulation of these critical metabolic activities has been the focus of recent investigation in tumor cell metabolism. Compelling evidence is emerging that oncogenes and tumor suppressor genes modulate the pentose phosphate pathway, fatty acid synthesis from glucose, anaplerosis and NADPH production activation in meeting these biosynthetic demands. Moreover, the common 'cancer pathways', including p53, RAS/MAPK, and the PI3Kinase pathways, that are dysregulated in glioblastoma, impinge at multiple points in the network of metabolic pathways. In particular, the PI3K/AKT/mTOR pathway appears to be a critical nodal point in tumor cell metabolism, through its effects on glycolysis and the production of lactate as well the ability to suppress macromolecular degradation in cancer cells (DeBerardinis, Lum et al. 2008). To date, most of the progress in understanding tumor metabolism has come from cell culture based studies using gain of function or loss of function mutations. However, significantly less is known about an individual tumor's metabalome in the context of its normal microenvironment. Investigation of the "metabolic phenotype" as the functional endpoint of a complex cancer genome and as a potential therapeutic target requires an in vivo model system that mimics the human tumor and a comprehensive understanding of genetic mutations that drive that specific tumor.

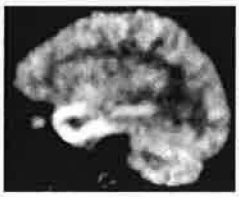
For more than 20 years, Dr. Craig Malloy's laboratory at UTSW has focused on developing and applying the stable isotope methods for the analysis of metabolic pathways in complex functioning systems. The development of these methods is important in basic biology for two reasons. First, classical radiotracer methods involving 14C and 3H are cumbersome because of the requirements for radiation containment and limited exposure in the laboratory. This constraint is widely understood. The second motivation is not widely appreciated. Conventional radiotracer methods provide very little information compared to the complexity of metabolic pathways in intact tissues. There is fundamentally a poor match between the information yield from radiotracers and the biological questions generated by complex systems such as glioblastoma. The use of stable isotopes with detection of products by NMR provides overwhelmingly greater information yield from any experiment, and is therefore the preferred method. Several years ago, Dr. Malloy and a group of investigators at UTSW interested in brain tumors and cancer metabolism (Ralph DeBerardinis, Juan Pascaul, Robert Bachoo, Changho Choi, Elizabeth Maher, Isaac Marin-Valencia) formed the Brain Tumor Imaging and Metabolism Group to study metabolism in the HOT model and in patients. It was expected that analysis of intermediary metabolism with stable isotopes would provide a comprehensive picture of metabolism in brain tumor patients and murine models that cannot be obtained by any other technology. The ability to follow the fate of glucose beyond a positive PET scan promised to be invaluable in understanding cancer metabolism. Moreover, the ability to use ^{13}C or other stable isotopes in patients and generate intermediary metabolic data in the setting of the tumor's normal microenvironment was a major and important leap forward in understanding pathological changes in metabolism.

^{13}C-Glucose infusion in patients having surgery

Pre-op Imaging

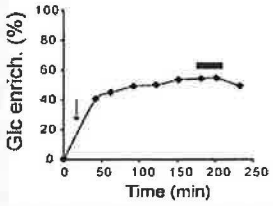
- 3T MRI
- 7T MRI
- ^{18}FDG PET





Surgery

- Infusion of ^{13}C-glucose



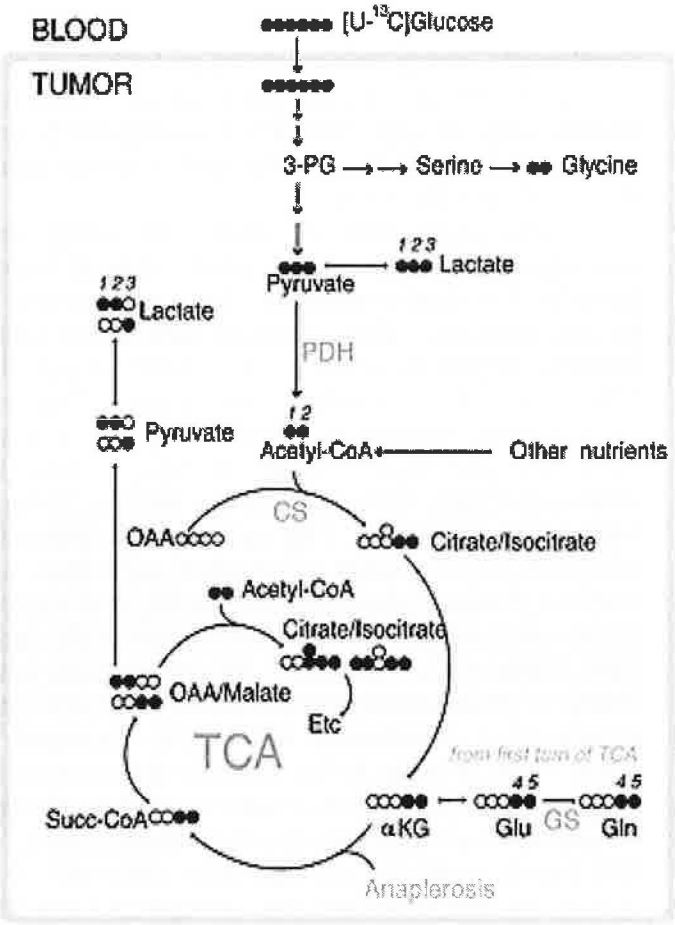
- Surgical navigation
- Biopsy & freeze-clamp tumor

Analysis

- ^{13}C and ^{1}H NMR
- Mass spec
- Molecular
- Histology

IRB approved August 2009

As shown above in the schema, uniformly labeled glucose ([U-^{13}C]glucose) was infused during surgical resection and tumor samples were subsequently subjected to ^{13}C NMR spectroscopy. The results of this work will be presented. Briefly, in a report of 11 tumors (9 gliomas and 2 brain metastases), analysis of tumor metabolites revealed lactate production as expected in each case (Maher, Marin-Valencia et al., 2012). However, it was also determined that pyruvate dehydrogenase, turnover of the CAC, anaplerosis and *de novo* glutamine and glycine synthesis were active and contributed significantly to the ultimate disposition of glucose carbon. Surprisingly, less than 50% of the acetyl-CoA pool was derived from blood-borne glucose, suggesting that additional substrates contribute to tumor bioenergetics. Metabolism reflects many clinically important aspects of tumor biology, including proliferative state, response to chemotherapeutic stress, and possibly the effects (Yazici, Sarialioglu et al. 2009)of particular oncogenes. Thus it is striking that the metabolic activities of eleven independent tumors were so consistent. Further work is necessary to determine whether individual driver



mutations are associated with specific metabolic features that could be used to predict prognosis or to individualize therapy.

This study illustrates a convenient approach that capitalizes on the high information content of ^{13}C NMR spectroscopy and enables the analysis of intermediary metabolism in diverse malignancies growing in their native microenvironment. In addition, this study demonstrated that the infusion of [U-^{13}C]glucose can be integrated easily into standard operating room procedures in patients undergoing surgical resection of high-grade gliomas and brain metastases. Adapting this protocol for analysis of systemic cancer is currently underway and it is anticipated that this protocol will become a common procedure in studying cancer metabolism.

3. Research Imaging: Unexpected new biomarkers and new hypotheses in low grade gliomas

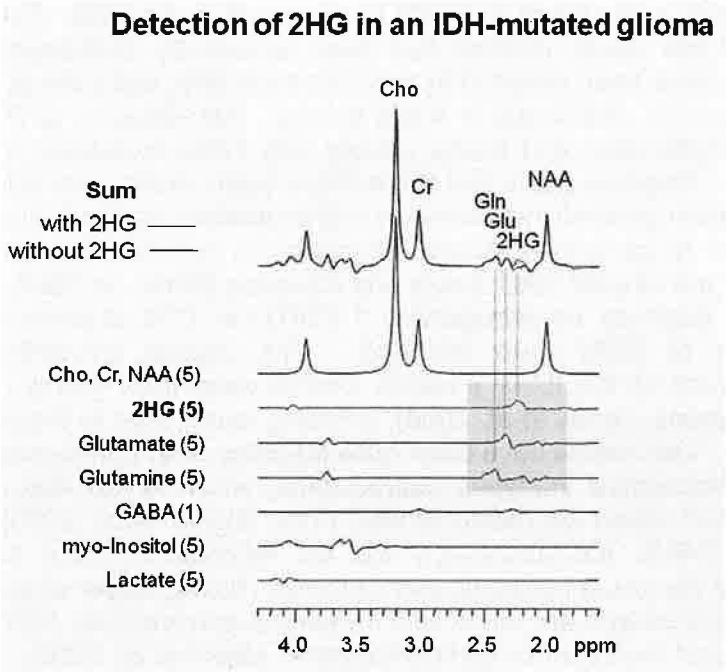
<u>The barriers to progress in management of low grade gliomas:</u>

Unlike the GBMs and brain metastases described above, the study of low grade gliomas is severely hampered by the lack of an animal model and lack of low grade glioma cells for in vitro study. As a result, research in this field has been at a standstill. For over 30 years, the genetic signature of low grade gliomas has been essentially unchanged. Identification of mutations in TP53 in have been reported in approximately 60% and LOH at 17p in ~40% as the two most common genetic alterations in these tumors. Amplification of PDGFRα is the most common reported amplification and tracks closely with TP53 mutations, but is seen in fewer than 20% of patients. Regional gains and losses have been catalogued using conventional and array-based comparative genomic hybridization and expression analysis has revealed a number of genes that appear to be overexpressed compared to normal brain although all generally infrequent (Kleihues and Ohgaki 1999; Louis and Cavenee 2000). In 2008, recurrent mutations in the active site of isocitrate dehydrogenase 1 (IDH1) in 12% of tumors in a genome-wide sequencing analysis of GBM were reported. The clinical annotation of the samples demonstrated that most of the IDH1 mutated tumors were from young patients who had a secondary GBM (Parsons, Jones et al. 2008), a finding which lead to the rapid investigation of IDH1 status in LGGs. The results have been quite stunning, with mutation rates exceeding 70% of LGGs in all the histological subtypes (astrocytoma, which is the vast majority, as well as oligodendroglioma, and mixed oligoastrocytoma) (Yan, Bigner et al. 2009). Similar rates are found in secondary GBMs, not surprisingly, but are detected in fewer than 10% of primary GBMs and in none of the grade I pilocytic astrocytomas (Balss, Meyer et al. 2008). Mutations in IDH2, the mitochondrial isotype are much less frequent (approximately 1-3%) but in most cases occurs in tumors without mutations in IDH1 (Hartmann, Meyer et al. 2009). These data suggest that IDH1 is an early event in gliomagenesis and, for the first time, implicates abnormal tumor metabolism in the pathogenesis of low grade gliomas.

The mutation in IDH1 occurs at arginine 132 and the homologous arginine in IDH2 (172). It has now been well established that mutant IDH1 catalyzes the NDAPH-dependent reduction of alpha-ketoglutarate to 2-hydroxyglutarate (2-HG) (Dang, White et al. 2009). 2-HG is of particular interest since it has been shown that children with L-2-hydroxyglutaric aciduria, an inborn error of 2HG metabolism, which leads to markedly elevated 2-HG levels have a increased risk for the development of malignant brain tumors, predominantly gliomas (reviewed in (Rogers, De Berardinis et al. 2010)). Thus, this metabolite appears to have oncogenic properties, contributing to the genesis and possibly progression of low grade gliomas. Prior to this discovery, the understanding of glioma metabolism in vivo related mostly to the finding that GBMs are "hot" on ^{18}FDG-PET scans while low grade gliomas are "cold". The marked uptake of glucose in GBM was thought to reflect the 'Warburg phenomenon', defined as excess flux of

glucose through anaerobic glycolysis with production of lactate despite an intact tricarboxylic acid (TCA) cycle. In contrast, uptake of glucose in LGGs is considered normal by ^{18}FDG-PET. It is unknown whether the change in metabolism simply reflects an increased rate of glucose utilization as a result of the marked increase in cellular proliferation or is a direct consequence of a molecular switch that governs the transition from LGG to GBM. With the identification of mutant IDH1 and 2 and production of 2-HG, it is clear that the transition to GBM represents a 'second step' in abnormal metabolism, with the first step being the metabolic consequences of 2-HG in LGGs, which are currently unknown.

^{13}C-glucose infusion in IDH-mutated gliomas in vivo, similar to the study described above is ongoing and is providing important insights into this subset of patients. In a study done in parallel, an IRB-approved imaging protocol has been ongoing in neuro-oncology since 2009, with the goal of imaging patients on the 3T and 7Tesla research MR scanners and working with the imaging scientists to improve detection and diagnosis. The results have been remarkable. To date, 102 patients have been enrolled and have multiple scans each. Data will be presented showing angiography on the 7T in GBM and the detection of 2HG by MR spectroscopy on the 3T(Choi et al, 2012).



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