Clinically Relevant Brain Tumor Model and Device Development for Experimental Therapeutics

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Abstract: This paper assesses the subcutaneous, orthotopic, and transgenic mouse models used to study glioblastomas (GBMs) as well as delineates our model to overcome the limitations of these currently used models. Subcutaneous model involves the injection of GBM cells into hind leg or back of a mouse, whereas in orthotopic model, the injection of GBM cells into the cranium of mice is required. Neither subcutaneous nor orthotopic models accurately display the infiltrative growth pattern of the tumor into the brain parenchyma characteristic of GBMs in humans. Transgenic models are achieved by pronuclear microinjection (into the male pronucleus, immediately after fertilization) or the injection of DNA into embryonic stem cells. Transgenic models are similar to human GBMs in every way, except they are not as genetically complex. To overcome the limitations in these models, we have developed a brain tumor model that exhibits all the histologic hallmarks of human GBM. We used a flank model initially to enrich a tumorigenic population of GBM cells from patient biopsies and a subsequent intracranial implantation to achieve the characteristics of tumors similar to those observed in human patients. The cells enriched by this method were then implanted and subjected to standard treatments such as chemotheraphy and radiation. Subsequently, we determined the treatment efficacy and rate of recurrence. Currently, we are using this approach to determine the treatment resistance pathways leading to recurrence and for developing a better combinatorial approach by short-circuiting the aberrant signaling pathways that are up-regulated in the treatment resistance tumors.

Keywords: Brain tumor, *In-vivo* model, Glioblastoma, Glioma stem cell and Radiation shield.

INTRODUCTION

Glioblastoma (GBM) is one of the most malignant and aggressive primary brain tumors. The tumors are heterogeneous and complex, with poor survival benefit despite various aggressive treatments including surgery, chemotherapy, and radiation therapy. Oftentimes, these tumor cells acquire resistance to chemotherapy and/or radiation therapy. They constitute 17% of all brain tumors and are not typically detected until they are in a late stage of growth. The median survival period from time of diagnosis is a dismal 9-15 months. The tumors are characterized by infiltrative growth patterns, sometimes centimeters away from the main tumor mass, into the brain parenchyma [1]. Thus, they present difficulty for surgical resection, radiation treatment, and chemotherapy. Before choosing one of the models to accomplish the objective of a given study, the advantages and limitations of each model should be evaluated. The current models used to study GBMs are subcutaneous, transgenic, and orthotopic. None of the three existing models can be used to completely mimic the growth of GBMs in humans due to their tumor heterogenity. Therefore, there is an unmet need for developing a unique GBM model. Therapeutic oncology is moving forward towards personalizing or individualizing treatments and it is important to develop a model that can adapt to the advances in the field. Hence, the current model we have developed to overcome the existing limitations in GBM models will be more suitable to test new experimental therapeutics.

EXPERIMENTAL

Subcutaneous Model

Perhaps the simplest existing murine model used to study GBMs is the subcutaneous model, and it is beneficial because it can reproduce the tumor easily. This model involves the injection of cancer cells into a hind leg or back of the mouse. Due to the proximity of the tumor to the surface and consequent high visibility, this model provides convenience for examining the rate of tumor growth and decline. The largest and shortest lengths of the tumor are considered when evaluating tumor volume. The most accurate volume calculations were obtained using the formula $V = W^2 \times L/2$ for caliper measurements. If tumor volume is measured using ultrasonography, the formula is: $V = (4/3) \times \pi \times (L/2) \times (L/2) \times (D/2)$, where V is tumor volume, W is tumor width, L is tumor length and D is tumor depth.

The radiosensitivity and the chemosensitivity of GBMs for different chemotherapeutic agents can be evaluated with subcutaneous models. These tumors are considered heterotopic. Benefits associated with subcutaneous models are easy reproducibility of tumor

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genesis and synchronicity. In addition, subcutaneous models produce tumors that are genetically secure [1]. Furthermore, some investigators use this model to study tumor tissue remodeling due to the fact that these tumors often display a high level of host-derived tumor interstitial molecules [2]. In subcutaneous models, the tumors typically grow in a compact form [1]. Another problem associated with subcutaneous models is the fact that the tumors often show necrosis during tumorigenicity, and therefore, are not stable. Finally, the microenvironment held by the mice injected with subcutaneous tumors is not similar to the microenvironment of GBMs in humans. Therefore, information gathered with subcutaneous models relating to metastasis and invasion is often irrelevant.

Orthotopic Model

Unlike subcutaneous tumors that typically grow in a compact form, the orthotopic model displays the infiltrative growth commonly found in human GBMs. The orthotopic mouse model is a somewhat recently developed model to study GBMs. This model requires the implantation of human GBM cells into the cranium of a mouse. Due to the fact that many opportunities for errors exist, a professional with adept surgical skills should perform the procedure in order to obtain the best reproducible results. In the majority of cases, sacrifice of the animal is required to observe the tumor growth in orthotopic models. In cancer cell lines that display fluorescence or luciference, three primary imaging methods are used to detect tumor growth: optical imaging, computerized tomography (CT), and magnetic resonance imaging (MRI). In comparison to ectopic tumors, orthotopic tumors display faster early-stage growth. In addition, glioma cells grown as orthotopic tumors display more similarity to each other in their microarray profiles than do subcutaneous tumors and *in vitro* cultures [3]. When the molecular subtype of GBM cells is evaluated using the Nearest Template Prediction Method, orthotopic xenograft models reliably keep the molecular subtypes of parental tumors [4]. These models display great similarity in their microarray profiles. The model also strongly resembles the human GBM progression process to the point that it can be considered clinically relevant. Consequently, most of the investigators consider orthotopic models to be the most clinically relevant of all the current models. However, orthotopic models possess limitations in their growth pattern. They do not always replicate the infiltrative growth in the brain parenchyma, which often characterizes human GBMs. They therefore, do not accurately represent the phenotype of the growing tumor, which can be detrimental to obtaining clinically relevant results.

Transgenic Model

Transgenic mouse models are an advanced method used to study GBMs because they contain artificially introduced foreign DNA in every cell which can be genetically manipulated. This is achieved by pro-nuclear microinjection (into the male pro-nucleus, immediately after fertilization) or the injection of DNA into embryonic stem cells. It may take multiple generations to achieve a fully transgenic mouse strain.

In order to be used effectively, transgenic models must satisfy several requirements. The mouse must contain the identical mutation expressed in human tumors, and the tumors should advance in similar preneoplastic stages that proceed in humans. Also, the mutation should be engineered within the endogenous locus (as opposed to being expressed as a transgene), and mutations should not be expressed until after postnatal development.

These models are beneficial for studying the progression of GBMs for multiple reasons. One primary advantage is that the model does not demand experimental involvement [5]. In addition, genes thought to be linked to the formation of tumors can be deleted, mutated, or overexpressed in the transgenic mice. Consequently, the mice are useful for studying the alteration of specific genes and the response of the tumor to various treatments over time. The mice also similarly model the human brain environment, replicating many important factors such as the blood-brain barrier. In addition, mice decline in a similar manner to affected humans, so they serve as beneficial models for studying the short- and long-term effects of GBMs. Transgenic mouse astrocytomas also have been found to mimic human astrocytomas in their increased vascularity, nuclear pleomorphism, infiltration, necrosis, and elevated mitotic index [6]. Unfortunately, transgenic mice models possess limitations such as their tumors do not typically exhibit the genetic complexity found in human GBMs.

RCAS (Replication-Competent ASLV long terminal repeat (LTR) with a Splice acceptor) Mouse Model: Over the last couple of decades, a number of groups have sought to identify molecular subtypes of gliomas, an undertaking that remains a critical challenge for glioma biologists [7-9]. Genetic alterations found in

adult gliomas have generally been divided into two groups. The first group contains genes that are related to growth factors, growth factor receptors, and signal transduction pathways. These include anomalies of platelet-derived growth factor receptors (PDGFR1/2) [10, 11], epidermal growth factor receptor [12], fibroblast growth factor receptor [13], ciliary neurotrophic factor [14], and hepatocyte growth factor/scatter factor [15]. The second group is comprised of genes implicated in cell cycle regulation, such as the *INK4a-ARF* locus which is altered in 60% of GBM [16], and the *RB* gene, which is altered in 33% of GBM [17]. Mutations in tumor suppressors *TP53* and *PTEN* are also common genetic alterations in human GBM [18]. In addition to the classification based on basic molecular alterations, GBM can be further subdivided into primary GBM, which arise *de novo*, and secondary GBMs that progress from lower-grade gliomas and likely constitute a highly heterogeneous group [7]. To study glioma development, gliomas modeled using the RCAS/*tv-a* system have been implemented to initiate tumors in newborn mice with known glioma oncogenic drivers. This model system allows somatic gene transfer of selected oncogenes such as *PDGF* and *Kras*, into targeted brain cells engineered to express the *tv-a* receptor. These transgenic *tv-a* mice can then be crossed with mice of various genetic backgrounds to model the effects of genetic aberrations such as tumor suppressor loss on glioma formation and response to therapy. Using this model, the authors have observed that oncogenes such as *Kras* and *PDGF* produce gliomas with higher grade and shorter latency in mice with *Ink4a-Arf-/-* and PTEN-loss backgrounds (mostly GBM) compared with wild type mice, which develop mostly low-grade tumors [19, 20]. These studies provide important mechanistic insights into the role of specific tumor suppressor loss in *PDGF*- and *Kras*-induced pediatric gliomas. The short latency of high-grade glioma development in these tumor suppressor-loss backgrounds results in developing GBM when they are very small (~10–15 g), which presents a challenge. However, this model is good for determining efficacies of various targeted therapies if the genetic background of tumor is known.

Our Model for Experimental Therapeutics

The study was conducted in accordance with OSU Intuitional Review Boards for IRB (2009C0065), IACUC (2009A0127) and IBC (2009R0169). The study consisted of two sections. We used flank models to verify the genomic integrity and enrich the tumorigenic population of cells. Next, we utilized intracranial models from the tumors obtained from the flank model to reproduce all the histopatholigical hallmarks of GBM. The main purpose of developing this model is to identify tumor treatment escape mechanisms and to discover new therapeutic targets that can be exploited for therapy in the future. In an effort to eradicate tumor treatment resistance, we tested drugs, investigational compounds, radiation treatment, and their combinations. Five animals were used for flank model and eight to ten animals for intracranial model for all the treatment arms. The large study group size was chosen in an effort to best obtain statistically significant results. A subcutaneous flank injection was performed to assess the proliferative rate and intracranial injection to evaluate the growth, niche specificity, invasiveness, and metastasis. A multitude of treatment strategies including radiation, gene silencing, small molecule inhibitors, and intracellular signaling cascade modulators were evaluated in the intracranial models to provide necessary physiological barriers and to exactly mimic the clinical setting.

Flank Model

The tumor tissue obtained from GBM patient biopsies after surgical resection and pathological review were used for isolating cells by enzymatic digestion. The immune compromised NOD-SCID mice were used as tumor carriers. A panel of isogenic cell lines that were used for previous studies were also used for the purpose of survival comparison. Following a two-week adjustment period, 5 mice per group were used to monitor the genetic integrity of tumor and specific growth characteristics.

After the enzymatic dissociation of tissues to single cells and filtration through cell strainer (BD Falcon Cell strainers 40 µm mesh size), the cells were spun-down at 1000 rpm at 4º C for 5 minutes, and the media was aspirated. The cell pellets were re-suspended and counted using cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA, USA) after staining with tryphan blue. Ten million viable cells in 100 µl of serum- free DMEM were injected into the right flank of NOD-SCID mice. The success rate of generating tumors from biopsies was 75%. The rate-limiting step is the time taken for implanting cells after surgery. The shorter the time (<5 h), the higher the success rate. For subsequent intracranial implantation, one of the criteria used was the time to tumor formation in flank model. Tumor volume doubling times range from 20 to over 50 days, with an average median doubling time estimated to be 30 days. The success rate in translating the flank

to intracranial tumor was 100% for tumors with the average median doubling time.

Initially, one animal was sacrificed after three to four weeks depending on tumorgenicity, followed by one additional animal after five to six weeks to monitor tumor growth. After fifteen weeks, the remaining mice were sacrificed. A NOD-SCID mouse bearing flank tumor is shown in Figure **1**. The tumorigenicity and genetic integrity were then verified. Throughout the latter period, the animals were monitored for their behavioral pattern, and their weight was recorded every three days. The tumor initiating cells were propagated again from the group of animals sacrificed after two to three weeks to serially passage them to verify genetic integrity following the same procedure previously mentioned. Cell lines derived from tumor biopsies were injected and key gene and epigenetic profiles were determined to ensure genetic integrity. After fifteen weeks, if there was no tumor burden, animals were euthanized.

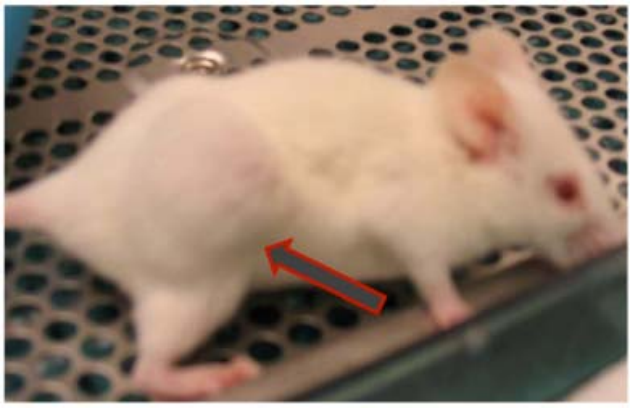

Figure 1: Flank Model. The tumors obtained from biopsies was enzymatically dissociated, implanted and grown in the flank of NOD-SCID mice.

Intracranial Model

We used the intracranial model to evaluate the relative treatment sensitivities. About 100,000 cells in a volume of 4-microliter medium were injected in the right frontal lobe of NOD-SCID mice. We planned to see if orthotopic tumors exhibit all the histopathological hallmarks of GBM. Next, we used the model to test the effect of drug and drug + irradiation on tumor progression. After observing the treatment response based on the time to develop tumor burden or symptoms after treatment, the mice were sacrificed to harvest the tissue and subjected to extensive analysis of tumors including genetic and proteomic profiling. This analysis has been a useful guide in determining the tumor treatment escape mechanism. As a next phase, if any of the identified resistance pathways are druggable and if FDA (Federal Drug Administration, USA) has approved the drug, we will add this new investigational compound to study the efficacy. The mutational landscape of tumors determines the treatment efficacy and survival benefit. Often the combination of drug + radiation approach may not be the best, this means that certain subsets of tumors will require only chemotherapy while certain others require radiation alone and the rest may require both chemotherapy + radiotherapy. Clearly, this one-treatment approach has to be changed. As we develop more personalized or individualized treatment, it is essential to identify these resistance pathways for standard treatment regimens and try to incorporate new investigational drugs, by using the genetic and molecular features of their tumors as a major determinant.

Surgical Procedure

The orthotropic implantation of tumors was carried out as follows. Pentobaribitol (40-50 mg/kg), ip (intraperotonially) was used for anesthesia. Once anesthetized, the head of the mouse was mounted on a KOPF stereotactic head frame securely with the aid of ear pins and a teeth stabilizer. The skin on the top of the head was washed with iodine solution. Aseptic surgical techniques were followed in accordance with the rodent surgical guidelines outlined by the ORRP (Office of Responsible Research Practice). All the axes in the stereotactic frame were set to zero degree on the head frame (vertical, horizontal, and anterior-posterior). Every three minutes throughout the procedure, the mice were monitored by observing their heart rate, jaw laxity, and reflexes. A small anterior-posterior incision through the scalp down to the level of the bone was made and the blunt end of the scalpel was used to further sweep away the scalp from the underlying skull. The junction of the coronal and sagittal suture lines (Bregma) was located and exactly 2 mm to the right a small burr hole was made with a small drill bit anterior to the coronal suture. After waiting for 3 minutes, stereotactic injection of the cells into the right frontal lobe using a 10 microliter Hamilton syringe was carried out as follows: the syringe tip was lowered into the burr hole 3 mm deep into the brain parenchyma, the brain was allowed to recover for 2 minutes, the cells were injected at volumes of 0.5 microliters at intervals of 15 seconds until the entire volume (2-4 microliters) was injected. After a wait-time of 2 minutes, the syringe was withdrawn slowly by 0.25 mm every 30 seconds until

the syringe was fully out of the brain. The slow withdrawal was to prevent the back-leakage of the injected substance along the needle tract. The burr hole was sealed with bone wax to occlude the bony defect, and the incision was closed with a surgical suture (4-0 vicryl with an rb-1 needle).

Post-operatively, mice were given 0.05 mg/kg buprenorphine subcutaneously every 12 hours for 3 days. Generally, after the recovery from anesthesia the mice behave well and active. The appearance and behavior of the animals that underwent surgery were observed in comparison with the control group. If there were any noticeable changes, the group that underwent surgery would then undergo a close follow-up and were moved, singly housed, and kept warm until they resumed their normal behavior. After 3-4 weeks, the tumors in the mice were imaged and the mice were randomized into groups. Treated animals were monitored daily for signs of discomfort and weight loss as a symptom of distress. During and after treatment, we looked for neurological symptoms including lethargy, poor feeding, paralysis, appearance of distress such as poor mobility, self-mutilization, hunched posture, dehydration, and skin ulcers. The follow-up MRI scans occurred immediately after treatment and again 4 weeks after treatment. Weight loss exceeding 20% (symptom of distress) resulted in euthanasia. After 25 weeks, the mice were sacrificed and tumors were dissected.

Imaging

The tumor volume and the time of treatment for animals were decided based on the MRI scans of animals. After treatment, the tumor growth delay, growth kinetics, and vascular volume fraction were computed using MRI scans. The MRI imaging of mice was performed on the 9.4 T system (Bruker BioSpin; Billerica, MA). The animals were anaesthetized with 2.5% isoflurane mixed with 1 liter per minute carbogen. They were maintained with 1-1.5% isoflurane. Physiologic parameters such as the ECG, respiration, and the temperature of the animals were monitored using a small animal monitoring system (Model 1025, Small Animals Instruments, Inc. Stony Brook, NY). If necessary, the ECG signal was obtained by placing subucutaneous ECG leads on the right forepaw and leg of the animal. The heart rate of the animal was maintained in the range of 350-450 beats per minute by adjusting the level of the anesthesia. A pneumatic pillow was used to monitor the respiration of the animal. Core temperature of the animal was monitored using a rectal thermometer and maintained at 37ºC using a warm air blower. For imaging, the animal was secured on the animal bed and placed in a birdcage resonator that was positioned at the correct anatomical location of the mouse in the MRI scanner. The animal remained in the resonator for approximately 30-60 minutes (10-30 minutes for initial calibrations followed by 30 minutes of data acquisition). If necessary, a gadolinium contrast agent (GdDTPA, 0.1 mmol/kg) was injected either intraperitonealy or intravascularly prior to MRI imaging. The animals were imaged three times: one time before treatment and twice after treatment. The animals were also scanned using MRI to calculate the tumor growth delay after chemoradiation. The irradiator RS2000 was used for radiating animals. The MRI imaging was recorded for the mouse before and after radiation and/or inhibitor treatment. The animals were imaged using MRI to measure the tumor size and vascular volume fraction (VVF) in selected mice in the treatment arms after randomization. Representative images of MRI scans are shown in Figure **2**.

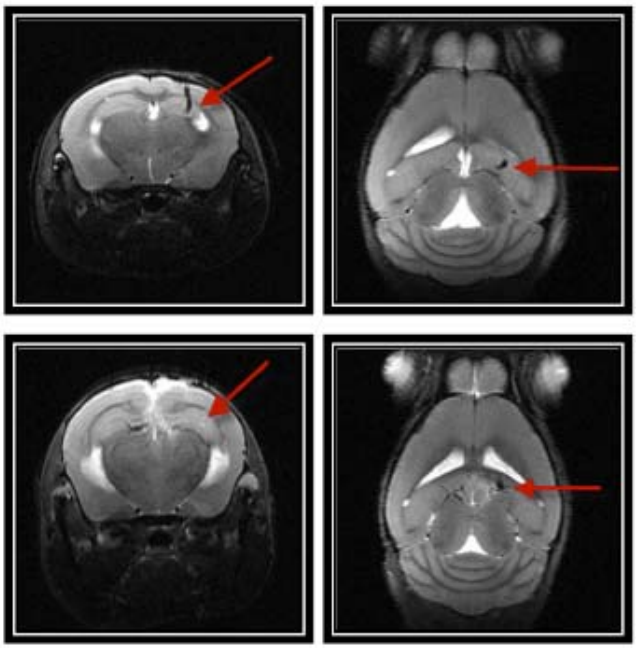

Figure 2: MRI scans of tumor. Magnetic resonance imaging (MRI) T1 and T2 of two NOD-SCID mice bearing tumors are shown. At this tumor volume we will start the treatment and follow-up studies will be conducted.

Drug and Investigational Compound Treatment

The drug we used for the study includes standard chemotherapeutic agents used for brain tumors such as temozolomide (TMZ), which have widely been used in clinical trials in the past decade. After 3-4 weeks, the mice were imaged to calculate the tumor volume and were randomized into groups: one to receive drug

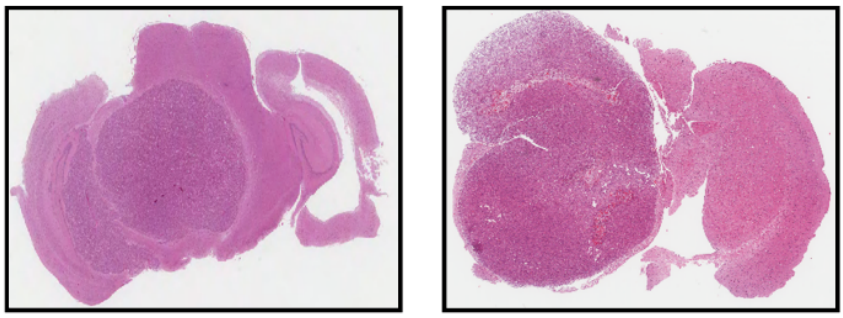

Figure 3: Our model. The flank tumors obtained were serially passaged by injecting the dissociated tumor cells at the right frontal lobe of NOD-SCID mice. The mice were sacrificed once they developed symptoms of tumor burden. The tumors obtained by this method exhibited all the histological hallmarks of GBM. The figure presents the H&E sections of two types of such tumors.

(100mg/kg), one to receive radiation treatment (RT) (5 fractions of 1Gy radiation dose), one to receive drug + RT (100 mg/kg and 5x1Gy),one to receive the investigational compound, one to receive the investigational compound + RT, and one to remain as a treatment-free control. The investigational compounds were identified after analyzing the tumor treatment escape mechanism. Depending on the nature of the drug/investigational compound, we administered the drug to the animal through intraperitoneal injection, intravascular injection, or intramuscular injection or po gavage. We identified FDA or NCI (National Cancer Institute) approved compounds to inhibit the gene or protein or pathway responsible for tumor escape to use for further studies. The animals had a close follow-up, and if we observed symptoms of drug and radiation induced toxicity, the mice were euthanized. Over the course of eight weeks, symptoms of neurotoxicity were observed and the mice were sacrificed. The brain sections were stained with Hematoxilin and Eosin (H&E) and were also stained for different antigens using immunohistochemistry techniques. The H&E sections for select glioma stem cell (GSC) tumors are shown in Figure **3**.

Radiation Treatment

One of the major issues while irradiating mice is sparing other parts of the body during radiation treatment of tumors. To combat this, we have developed a radiation shield that one can use for radiating tumor diameters ranging from 3 mm and above in order to avoid the whole brain radiation and radiation to other organs. The radiation shield is shown in Figure **4**. The irradiator RS2000 OR GAMACEL RADIATOR was used for radiating animals using the calibrated and specially designed lead shield. The MRI imaging recorded for the mice was used to mount the animals in the radiation shield. Animals were anesthetized and mounted in the lead cage with a narrow hole for irradiation, which can be adjusted based on the tumor volume. The animals were secured for a period of 30 minutes during the irradiation procedure. Over the course of eight weeks, symptoms of neurotoxicity were observed and affected mice were sacrificed.

Study Endpoint and Euthanasia

After 4-16 weeks, we looked for neurological symptoms and excessive (20%) weight loss. Affected mice were sacrificed. After 16-20 weeks, the mice were sacrificed and tumors were dissected. Euthanasia was performed by CO_2 inhalation. The animals were monitored until they were no longer breathing and were unresponsive to noxious stimuli, such as a toe/tail pinch. We performed cervical dislocation of the unresponsive animals to confirm death. Brains were removed, tumor sections were immunostained, and nucleic acids were isolated for further studies to assess the genomic markers needed to ascertain genomic integrity, tumorogenicity, and target treatment (TT) sensitivity. The follow-up MRI imaging were taken after treatment to compute the tumor growth delay, growth kinetics, and vascular volume fraction. By way of our *in vitro* studies such as effect of radiation on DNA damage, we assessed the treatment resistant sub-population and then used them for a further detailed study using *in vivo* models to identify novel therapeutic targets, which could sensitize these cells towards existing treatments.

Experimental Therapeutics

We have utilized our GSC model to study the efficacies of different therapies. The mice were implanted with GSCs and monitored for early symptoms of tumor burden by MRI imaging. At a pre-determined tumor volume, the mice were treated with chemotherapeutic agents and radiation. Representative

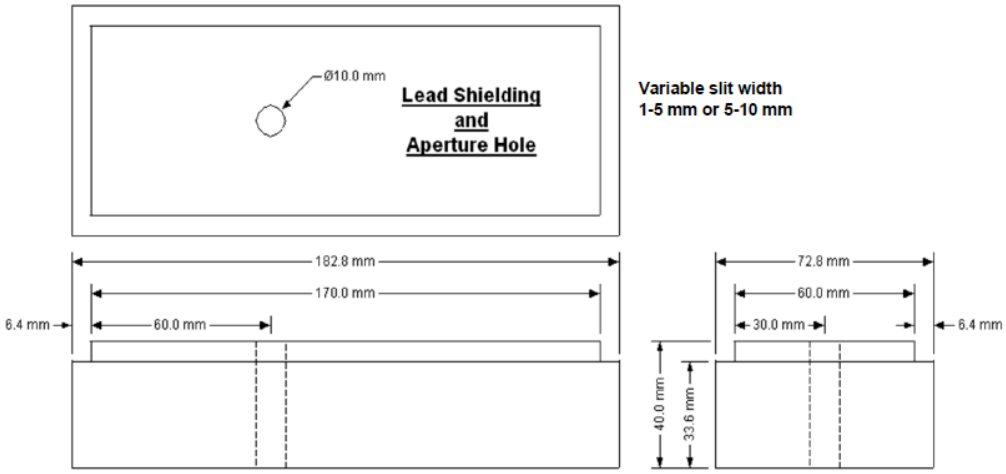

Figure 4: Radiation Shield. Design aspect of radiation shield used for shielding other organs of NOD-SCID mice while radiating tumors. Mice will be secured inside the device after anesthesia, the tumor location will be oriented with the aperture hole and the mice will be subjected to radiation treatment.

H&E- and Ki-67 staining of treatment responsive and resistant tumors are shown in Figure **5**.

CONCLUSION

This work shows that the flank and subsequent orthotopic xenografts generate tumors similar to patients with a high success rate. Furthermore, we have also shown that these models could be used to predict the outcome of various drugs and radiation treatments. The success rate of generating GBM cells and GSCs by this approach was higher compared to culturing them directly on plastic dish (*in vitro*). We have taken this approach in our laboratory for close to a decade, and our observation is that the flank and the tissue niche are more suitable to enrich cells that exhibit all the histological hallmarks of GBM. We previously reported that neurosphere cultures isolated from human GBM specimens were enriched for GSCs that could self-renew and efficiently generate orthotopic tumors in immunodeficient mice [21]. The GSCs generated highly invasive tumors with hypervascularity and intratumoral bleeding. Thus, the GSCs isolated in this way can display varying degrees of invasiveness and can replicate the pathological features of the patient. This ability of GSCs to replicate the pathological hallmarks of GBM provides a preclinical GBM model potentially representative of the disease.

Most human gliomas are characterized by diffuse infiltrative growth in the brain parenchyma. Partly due to this characteristic growth pattern, gliomas are notorious for their poor response to current therapies. However, many animal models for human gliomas do not display this diffuse infiltrative growth pattern. Furthermore, there is a need for glioma models that represent adequate genocopies of different subsets of human gliomas (e.g., oligodendrogliomas). We have

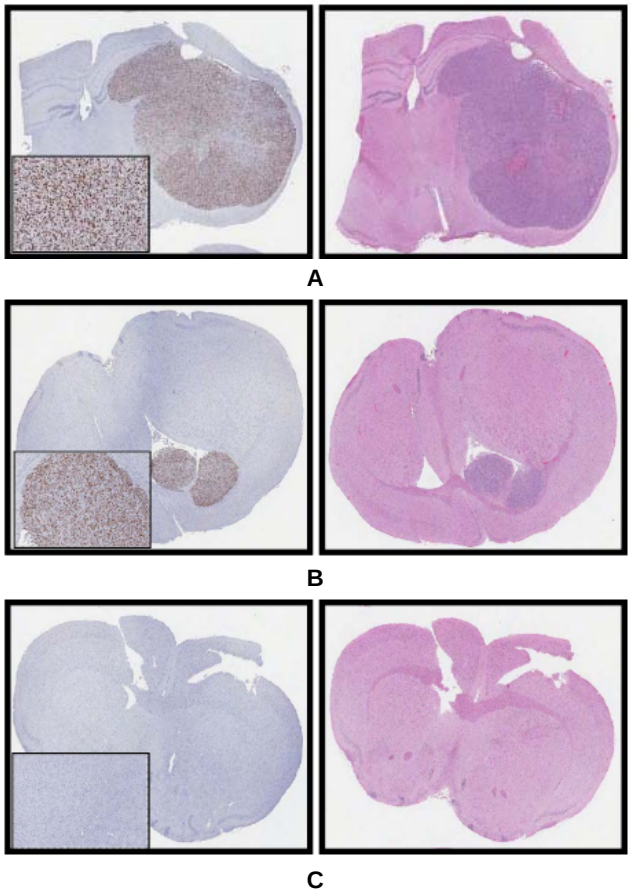

Figure 5: H&E and Ki67 stained treatment responsive and resistant tumors using the coronal section from the brains of NOD-SCID mice. (**A**) Control tumors that were not treated. (**B**) Tumors treated with drug (temozolomide) that show moderate tumor shrinkage and a decrease in tumor burden. (**C**) Tumors treated with radiation and drug that show almost no signs of residual tumor.

developed GSC xenografts that replicate the distinctive cytological hallmarks and diverse histological variants associated with the corresponding patient GBM. We anticipate this type of model could be extensively used for individualizing glioma therapy and in obtaining a better treatment outcome in individuals affected with this devastating disease.

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