Background

Glioblastoma (GBM) is the most common malignant primary adult brain tumour, and is highly aggressive and resistant to standard therapies. Feared for its near uniformly fatal prognosis, this astrocytic tumour (WHO Grade IV) is characterized by extensive cellular and genetic heterogeneity (1-2). Despite advances in multimodal therapies involving surgical resection, craniospinal irradiation and high-dose chemotherapy with the alkylating agent temozolomide (TMZ), the median survival is <15 months with <10% of patients surviving for two years without relapse (3). The Cancer Genome Atlas (TCGA) project pioneered the stratification of GBMs into Mesenchymal, Proneural and Neural subtypes through investigation of individual genomic and transcriptomics (4-6). This has provided great insight into genetic regulation of GBM and patterns of gene expression that have been collated to identify molecular subgroups with putative prognostic or predictive significance. Many genes involved in control of proliferation, cell cycle and apoptosis have been implicated in GBM pathogenesis including EGFR, TP53, MDM2, PTEN and PDGFR (7-9). Although there have been major advances in understanding the molecular genetic alterations of GBM, it was unclear as to whether all cells within this heterogeneous tumour were equivalent in their ability to maintain tumour growth.

Research Problem

The impact on treatment and prognosis has been limited by the fact that the genetic landscape of tumours is continually evolving spatially and temporally, generating extensive cellular complexity and heterogeneity within a single tumour (10-13). At the cellular level, resemblance between stem and cancer cell self-renewal mechanisms have indicated that a small fraction of tumour cells, termed brain tumour initiating cells (BTICs), present increased self-renewal capacity, proliferation, differentiation potential as well as the ability to recapitate human tumour biology when injected into immunocompromised mice (14-20). GBM heterogeneity, subsequent poor patient outcome and tumour relapse can be explained by multiple subpopulations of BTICs and their self-renewal and chemoradiotherapy evasion properties (2, 19, 21-23). The factors that maintain the relative balance between self-renewal and differentiation are likely dysregulated in cancer, and many of the key signaling pathways that are active during brain development are disrupted in brain tumorigenesis (9, 24-26). One such factor implicated by our preliminary data and by the literature is the chromatin-modifying Polycomb-group gene Bmi1 (27). Upregulation of the self-renewal property in distinct clonal cells via the Bmi1 oncogene at diagnosis is also involved in GBM tumour growth and sustainability. Bmi1 overexpression is highly associated with poor clinical outcome in GBM and other human malignancies such oral, esophageal, prostate, pancreatic, neuronal, non-small cell lung, breast cancers and hepatocellular carcinomas. Inhibition of Bmi1, however, has shown to be efficacious in impairing stem cell self-renewal and reduced tumourigenesis (20). Recent genomic profiling of GBM has shown that clonal evolution within the tumour may progress through standard therapies such that GBM recurrence may no longer resemble the genetic landscape of the diagnostic tumour (3, 28-29). Intratumoral heterogeneity (ITH) at the cellular, genetic and functional level has been shown to occur to a startling degree in many cancers, and is increasingly appreciated as a key determinant of treatment failure and disease recurrence (29-30).

Hypothesis

We hypothesize that each cell within the tumour has variable proliferative, self-renewal, and differentiation ability, in which BTICs hold the greatest capacity to reform tumours. Characterization of novel therapeutic agents, including Bmi1 inhibitors, in combination with current therapy to target BTICs may not only elucidate signaling pathways vital for tumour progression and recurrence in GBM, but also drive innovative treatment options for these terminally ill patients.

Specific Aims

Aim 1: Functionally profile the effects of small molecule inhibitors against Bmi1 through a multitude of in vitro assays.

Aim 2: Validate the inhibitor with the greatest in vitro efficacy in established mouse adapted patient-derived therapy models via inhibitory agent mono- and combinatorial therapy with Standard of Care (SoC) at various time points throughout tumour progression.

Aim 3: Perform a genome-wide unbiased CRISPR screening on treatment-refractory GBM in order to identify therapy-resistant genes, to then co-target Bmi1 and top hits from the screen by combination of small molecule inhibitors.
Methodologies

**Aim 1:** To study the functional effects of Bmi1 reduction in GBM recurrence, an initial dose response study will be conducted in multiple GBM cell lines. Once an effective therapeutic dose is established, a variety of *in vitro* assays will be performed including: (i) dose response curve with 2-fold dilutions of Bmi1 inhibitors ranging from 20µM to 39nM to determine the concentration at which half of the treated cells die; (ii) mixing experiment to validate that such Bmi1 inhibitors spare normal neural stem cells (NSCs), such that NSCs and GFP-tagged patient-derived GBM cells are treated with an inhibitor, at a concentration relative to GBM cells, mixed and evaluated based on GFP levels; (iii) western immunoblotting to confirm a reduction in Bmi1 protein levels; (iv) RT-qPCR of Bmi1 downstream targets in GBM cells to show that such genes are not affected by the presence of Bmi1 inhibitors; (v) secondary sphere formation and limiting dilution assays to identify the effects of small molecule inhibitors on self-renewal capacity of treated cells; (vi) profile stem cell markers, such as CD133, CD15 and Bmi1 via *flow* cytometry to identify the percentage of BTIC subpopulations in treated cells; (vii) cell cycle analysis *via* flow cytometry to determine if inhibitor treated cells lose their uncontrolled proliferative potential and arrest in S phase.

**Aim 2:** We have uniquely modified the existing chemoradiotherapy protocol in GBM patients for treatment of NOD-SCID mice engrafted with GBM BTICs. Our *in vivo* mouse-adapted therapy model has the distinct advantage of generating recurrent, human, treatment-refractory GBM. With identification of the most promising small molecule Bmi1 inhibitor, we will further test its ability to reduce tumorigenic potential of GBM cells *in vivo*. Intracranial injection of GBM will be followed by an *in vivo* treatment regiment with a small molecule inhibitor targeting Bmi1. Bmi1 inhibitor alone, vehicle alone, SoC (2 Gy cranial irradiation and 50mg/kg TMZ), and SoC, along with Bmi1 inhibitor. Tumour burden will be evaluated with magnetic-resonance imaging (MRI) and immunohistochemistry (IHC) through collection of mice brains at various time points: (i) engraftment, (ii) post-radiation, (iii) post-chemotherapy, (iv) post-inhibitor treatment, (v) post-combinatorial therapy and (vi) recurrence. Other organs will be collected for pharmacokinetic/pharmacodynamic (PK/PD) studies to investigate toxicity.

**Aim 3:** We will perform functional CRISPR knockout (KO) of putative drivers of treatment-refractory GBM stem cells transfected with lentivirus containing CRISPR KO constructs against the top selected genes from CRISPR screening, in order to test the role these genes play in driving treatment-refractory disease. The effects of knockout will be evaluated using established stem cell assays indicated in Aim 1, along with testing such samples through the *in vivo* protocol outlined in Aim 2 with greater emphasis on the combinatorial cohort. Genes from the screen will be resistant to SoC, therefore we will identify and co-target such genes in a subsequent *in vivo* trial.

Hypothesized Results

**Aim 1:** *In vitro* experiments will further elucidate Bmi1 inhibitors to sufficiently diminish protein levels of the target in preclinical models.

**Aim 2:** Combinatorial *in vivo* model with a Bmi1 inhibitor holds greater potential for improved survival benefit and tumour burden reduction, as SoC will be able to attack the bulk tumour, while treatment with a small molecule inhibitor will further target the BTIC population in GBM recurrence.

**Aim 3:** Analysis of CRISPR screens on GBM BTICs will lead to identification of dysregulated genes and pathways that govern tumour recurrence with great prospect of developing a treatment modality, more efficacious than standard therapy.

Rationale for Proposed Research/Therapeutic Relevance

To address the complexity of GBM heterogeneity and failure of conventional treatment, we have created a multi-disciplinary proposal to merge clinically relevant and novel models of GBM. With standard therapy, taking an unsuccessful mono-modal approach in completely eradicating all tumour sample to such clonally divergent diseases suggests the dire need for combinatorial therapies with potent inhibitory agents. In addition, it promotes collaborative industry partnerships. Our proposal aims to validate novel therapeutic targets that selectively reduce self-renewal capacity in BTICs that have been postulated to be the main contributors to the aggressive nature of GBM. Overall, this alludes to the idea that each cell within the tumour has variable proliferative, self-renewal, and differentiation ability, in which BTICs hold the greatest capacity to reform tumours. Selective targeting of these cells may provide avenues for de-escalation of harsh therapeutic regimens, while elucidating signaling pathways vital for brain tumour progression and recurrence. As there are no proven or effective clinical treatment options for patients with relapsed GBM, our proposal is all the more clinically meaningful and consequential.
References


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