

REVIEW

Intratumoral heterogeneity: pathways to treatment resistance and relapse in human glioblastoma

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Intratumoral heterogeneity (ITH) has increasingly being described for multiple cancers as the root cause of therapy resistance. Recent studies have started to explore the scope of ITH in glioblastoma (GBM), a highly aggressive and fatal form of brain tumor, to explain its inevitable therapy resistance and disease relapse. In this review, we detail the emerging data that explores the extensive genetic, cellular and functional ITH present in GBM. We discuss current experimental models of human GBM recurrence and suggest harnessing new technologies (CRISPR-Cas9 screening, CyTOF, cellular barcoding, single cell analysis) to delineate GBM ITH and identify treatment-refractory cell populations, thus opening new therapeutic windows. We will also explore why current therapeutics have failed in clinical trials and how ITH can inform us on developing empiric therapies for the treatment of recurrent GBM.

Key words: glioblastoma, intratumoral heterogeneity, brain tumor initiating cells, recurrence, models of resistance, polytherapy

Introduction

Glioblastoma (GBM), a highly aggressive astrocytic tumor (WHO grade IV), is the most common primary brain tumor in adults [1, 2]. Despite multimodal therapy consisting of surgical resection, radiation, and chemotherapy with the alkylating agent temozolomide (TMZ), the disease rapidly progresses and leads to relapse at 8–9 months post diagnosis, with an average survival of only 15 months [3–5]. This poor prognosis for GBM has been attributed to extensive cellular and genetic heterogeneity existing not only between patients, but also at an intratumoral level [6–9]. A molecular GBM classification by The Cancer Genome Atlas (TCGA) has offered insights into genetic regulation of GBM with identification of molecular subgroups with putative prognostic significance [10, 11]. The four subgroups of GBM described by TCGA, namely classical, neural, pro-neural and mesenchymal, were identified using transcriptional profiling data of bulk tumor specimens and based on dominant genes expressed in each group [11]. The classical subgroup is marked by amplifications or mutations in the epidermal growth factor receptor (EGFR); the neural subgroup is characterized by expression of neuronal genes; the

pro-neural subgroup expresses neural stem cell genes such as Sox2 (sex determining region Y-box2) and Olig2 (oligodendrocyte transcription factor 2) and is driven by platelet derived growth factor receptor alpha (PDGFRA) signaling; and the mesenchymal subtype is distinctly identified by mutations in the neurofibromatosis 1 gene. Despite clearly distinct transcriptional profiles of the four subgroups of GBM, the clinical prognosis of each subgroup remained the same with only a slight survival advantage of aggressive chemoradiotherapy for the pro-neural subgroup. Secondary GBMs are tumors that progress from a pre-existing low-grade glioma to GBM and largely fall into the pro-neural subgroup. These secondary GBMs are characterized by mutations in IDH1 and 2 (isocitrate dehydrogenase) as well as upregulated PDGFRA signaling. Despite extensive genomic and transcriptomic profiling of GBM by the TCGA to delineate molecular groups, most tumors were found to harbor alterations in common oncogenic pathways [receptor tyrosine kinase (RTK) signaling through mutations/amplifications in receptors such as EGFR and PDGFRA; mutations in downstream partners of Akt pathway such as PI3K and PTEN; apoptosis signaling through

mutations in p53; and cell cycle control signaling through alterations in CDKs] [10, 12]. Overall, the impact on treatment and prognosis of GBM subgroups has been limited by the fact that the genetic landscape of tumors is continually evolving through space and time [13–15], generating an almost unimaginable degree of cellular complexity and heterogeneity within a single tumor [16–18]. Such intratumoral heterogeneity (ITH) is increasingly believed to be one of the key determinants of therapy failure in GBM.

Intratumoral heterogeneity in GBM

Although the classification of GBM into four distinct molecular subgroups by TCGA attempted to address the challenge of heterogeneity in GBM [11], recent studies show that the GBM subgroups are flexible and vary spatially and temporally within the same tumor. A study by Patel et al. [9] showed that at single cell RNA-sequencing resolution, a single tumor consisted of a heterogeneous mixture of cells representing all of the different GBM subgroups. When examining the pro-neural subgroup, which had the best survival of all GBM subgroups, the authors showed that patients with pro-neural tumors that also bore markers of other subgroups had poorer survival, especially if the relative representation of the alternative subgroups was high in the tumor [9], emphasizing the role ITH may play in therapy resistance. Another study by Reinartz et al. [19] show that single cell derived GBM subclones have distinct genetic identity and maintain differential drug resistance profile. Initial reports of ITH in GBM identified coactivation of multiple RTK such as EGFR, Met and PDGFR, which required poly-targeting approach of RTKs to abrogate downstream signaling and cell viability [20]. Similarly, Szerlip et al. [13] showed heterogeneous amplification of EGFR and PDGFRA within GBM cell subpopulations. Inhibition of both RTKs was required to attenuate the activity of downstream target PI3K (phosphoinositide-3-kinase) and inhibit tumor growth. Additionally, multiple aberrations in EGFR identified through single-cell genome sequencing, have been found to co-exist in GBM and in fact some EGFR variants (EGFRvIII and EGFR carboxy-terminal deletions) tend to exist in mutually exclusive subclonal populations [21]. Although factors such as CNS penetration of agents, target selection and limitations in patient selection based on biomarker presence also contributed to therapy failure with RTK inhibitors in clinical trials, the observation of extensive ITH in GBM suggests the need for combinatorial therapies to address the challenge of therapy failure.

Further clouding the molecular subgrouping of GBM is the idea of spatial heterogeneity, which confounds our diagnostic and therapeutic efforts since previous genomic studies relied on a single regional biopsy to subgroup a patient. By sampling geographically distinct regions of single tumors, Sottoriva et al. [14] showed that genome-wide GBM ITH can be decomposed to reveal spatial and temporal tumor evolution, and based on gene expression levels, tumor fragments from the same patient may be classified into different GBM subgroups. These studies together inform not only on the extensive genomic heterogeneity that exists in GBM, but also present heterogeneity as a possible asset to evade therapy and generate resistance (Table 1). Consequently, ITH may then give rise to subclonal populations of cells with

selectable traits that can respond to and escape any given stress, including therapy [22].

Intratumoral heterogeneity in recurrent GBM

From an evolutionary perspective, the divergent development of subpopulations of cancer cells within the same tumor is likely at the root of therapy failure, the development of treatment resistance, and ultimately, recurrence of the malignancy (Figure 1). A study by Johnson et al. [23] showed that low grade gliomas and their paired recurrences only shared a few early mutations and were highly divergent. They also found that in 43% of profiled GBM cases, at least half of the mutations in the initial tumor were undetected at recurrence, suggesting that therapy acts as a selection pressure or bottleneck for tumor evolution from minority cell populations present at the time of initial diagnosis. Moreover, they also discovered that therapy might in itself drive the emergence of treatment-resistant subclones, as secondary GBMs from low-grade gliomas were found to be hypermutated and bearing a TMZ-induced mutagenesis signature.

The clonal evolution of primary GBM to recurrence was further demarcated through whole-genome and multisector exome sequencing studies of primary GBM and matched recurrences, which suggested both clonal and ancestral origins of GBM recurrence after therapy [24]. Kim et al. confirmed that while some GBM recurrences bore ancestral p53 driver mutations detectable in the primary GBM, many other recurrences were driven by branched subclonal divergent mutations not present in the primary GBM [24]. A case study by Favero et al. [15] again showed that the driver clonal mutation in a primary GBM was lost in the recurrence, which itself was dominated by a subclone from the primary GBM. Another study of the spatiotemporal evolution of the primary GBM epigenome to recurrence further consolidated the extent to which genomic instability and ITH are driven by therapy [25]. Further studies to explain patterns of GBM recurrence discovered that a spatially local recurrence of GBM was marked by a high retention of initial tumor mutations, following a linear evolution model, while a spatially distant recurrence retained fewer mutations from the initial tumor and followed a branched evolution model of recurrence [26]. In the recent study by Wang et al. [27], which comprises the largest longitudinal analysis of both genomic and transcriptomic data from GBM patients through therapy, the authors again show that 63% of patients change expression-based subtyping. In addition, they identified mutational landscapes that corresponded exclusively to the primary or the recurrent GBM as well mutations shared between the two. Interestingly, EGFR and EGFRvIII, both common targets for clinical trials, were largely reserved to the initial tumor and not the recurrence. Their data also suggest that the evolutionary divergent cellular populations that seed relapse existed years before diagnosis. By determining how both genetic and epigenetic events are clonally selected during GBM progression and constructing phylogenetic and phylo-epigenetic trees of GBM patients at diagnosis and recurrence, these studies documented both linear and branched divergent subclonal evolution, suggesting that targeted monotherapies based on the tumor genome at diagnosis are doomed to failure [28].

Table 1. Characterization of intratumoral heterogeneity in GBM in the past decade

Primary author	Publication year	Methodology	Samples	Key findings
Stommel	2007	<ul style="list-style-type: none"> • RTK antibody array • Single cell immunofluorescence 	Glioma cell lines and primary GBM	<ul style="list-style-type: none"> • Co-activation of multiple RTKs including EGFR, PDGFRA and MET • Single GBM cells co-express activated RTKs • Co-targeting of multiple RTKs abrogates PI3K signaling
Snuderl	2011	<ul style="list-style-type: none"> • FISH analysis 	Archived GBM specimens	<ul style="list-style-type: none"> • Heterogeneous amplification of EGFR, PDGFRA and MET • Different regions of tumor represent differential RTK amplifications
Szerlip	2012	<ul style="list-style-type: none"> • FISH analysis 	Frozen GBM	<ul style="list-style-type: none"> • Coamplification of EGFR, PDGFRA and/or MET is not mutually exclusive in single cells
Sottoriva	2013	<ul style="list-style-type: none"> • Copy number arrays • Gene expression arrays 	Multiple sampling from each GBM	<ul style="list-style-type: none"> • Different regions of a brain tumor harbors different aberrations and alterations in gene copy numbers • Different fragments present distinct transcriptional profile and are classified into different GBM subgroups
Patel	2014	<ul style="list-style-type: none"> • Single cells RNA sequencing 	Primary GBM	<ul style="list-style-type: none"> • Single cells from the same tumor present distinct transcriptional profile and can be categorized in different subgroups • Tumors present with a gradient expression of stemness-related genes at a single cell level
Francis	2014	<ul style="list-style-type: none"> • Single cell whole genome sequencing 	Primary GBM	<ul style="list-style-type: none"> • Multiple mutations within the same gene coexist in GBM • Gene mutation variants can exist in mutually exclusive cell populations
Kim	2015	<ul style="list-style-type: none"> • Whole genome sequencing • Multi-sector exome sequencing 	Primary and recurrent GBM	<ul style="list-style-type: none"> • Recurrence follows two modes of evolution: 1. Linear, where recurrence shares extensive genetic profile with the primary; 2. Divergent, where recurrence branches off from an earlier ancestor of the tumor and shares fewer genomic similarity with the primary tumor
Meyer	2015	<ul style="list-style-type: none"> • Single-cell derived clonal population 	Primary and recurrent GBM	<ul style="list-style-type: none"> • Functionally heterogeneous cells exist in single GBM • Single-cell derived clonal populations exhibit different MGMT promoter methylation status leading to differential response to TMZ • TMZ-resistant cells pre-exist in primary GBM specimens

Meta-analysis of all recent clinical trials for GBM patients has also predicted the failure of monotherapy to target the now well documented complexity of ITH in GBM [29], stressing the importance of multimodal therapy whenever clinically feasible, and highlighting the need to develop innovative and informed polytherapeutic strategies for this highly complex disease. The sum of the recent emerging literature on ITH and GBM, including single cell sequencing studies and longitudinal genomic profiling of GBM progression, has mapped multiple iterations of the clonal hierarchies that exist in GBM, and it is clear that spatial and temporal evolution are at play. However, whereas some models suggest that truncal mutations present in the primary tumor, such as PI3KCa or IDH mutations or FGFR-TACC3 fusion events, may inform therapies more effectively than private events such as EGFR amplifications which are exclusive to only a

few regions of the tumor [30], other models suggest that subclonal divergent events present exclusively at recurrence (arising either from rare clones that are not detected in the primary tumor or from mutational events that arise only after chemoradiotherapy) warrant a closer examination of the recurrent tumor to find efficacious therapeutic targets [23, 27]. In the end, the pattern of clonal evolution will likely vary from patient to patient, and only large population-based studies of the clonal maps of hundreds of sequenced GBMs will eventually discern reproducible cohorts of patients that recur in a similar manner. In any case, intratumoral genetic heterogeneity in clonal cell populations represents the root of therapy failure, the driver of development of treatment resistance, and ultimately results in recurrence of the malignancy.

A recent study by Meyer et al. [31] demonstrated that clonal populations derived from single cells have a variable response

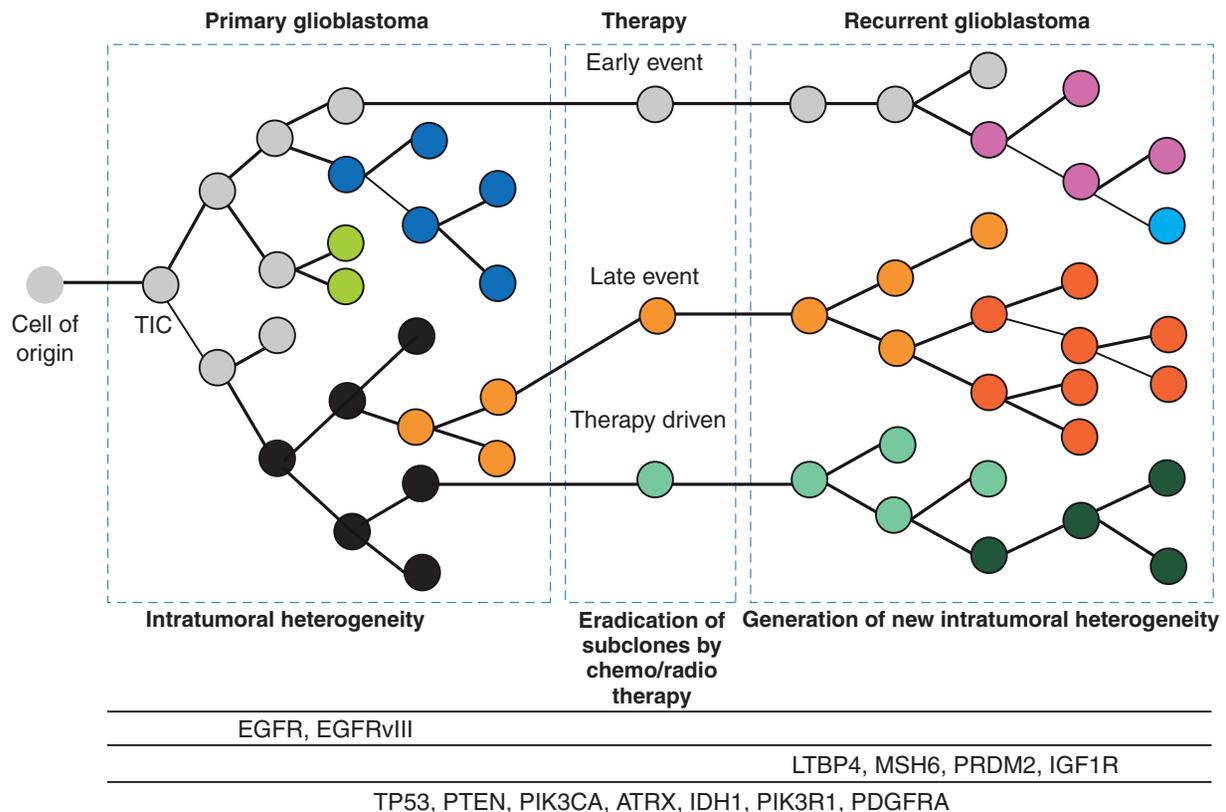


Figure 1. Subclonal populations in primary glioblastoma escape therapy and give rise to treatment-refractory, heterogeneous recurrent glioblastoma. After a normal cell acquires mutations (black outlined circles), it expands into multiple subclonal populations of glioblastoma with selectable traits against any stress (represented by different colored circles), including therapy. The administration of therapy for primary glioblastoma, leads to the selection of subclonal cell populations (early event subclone or late event subclone) or gives rise to a therapy-driven-resistant subclone. These treatment-refractory subclonal populations then seed tumor relapse and lead to the formation of a heterogeneous recurrent glioblastoma that has a distinct clonal composition from primary glioblastoma. Mutations in multiple genes have been identified to be specific to either the primary GBM or the recurrent GBM as well as those common to both.

TMZ as well as other drugs, linking genomic heterogeneity to functional heterogeneity. These single-cell derived clonal populations also presented with differential EGFR expression and O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status, a biomarker for TMZ resistance. Furthermore, study by Parker et al. [32] showed that ITH is not only evident for MGMT promoter methylation but also for several other genes of the DNA repair pathways, which could explain discordance in MGMT promoter methylation status and response to TMZ in some patients with GBM. Similar reports of clonal populations derived from single GBM cells show distinct phenotypic and proliferative characteristics in both *in vitro* and *in vivo* model systems [19, 33]. These studies suggest that functional heterogeneity in GBM is not only a derivative of genetic mutations but epigenetic mechanisms might also be playing a role, as cells from a single genetic background showed diverse expression of important GBM genes and differential functional response to drug treatment. At the cellular level, functional GBM heterogeneity can then be explained by the existence of multiple cellular subpopulations of cancer cells that have acquired stem cell properties of self-renewal and multi-lineage differentiation, variably labeled in the literature as BTICs (brain tumor initiating cells) or GICs (glioblastoma initiating cells) [34–37].

Brain tumor initiating cells may drive GBM recurrence

BTIC models [35, 38] combined with genomic deep-sequencing technologies have begun to resolve the extent of ITH in GBM. BTICs may arise from the dysregulation of genes that govern self-renewal, the cardinal property of stemness that allows a stem cell, at each cell division, to generate an identical copy of itself and a cell of the same or different phenotype [39]. The BTIC model of GBM is thought to recapitulate the functional heterogeneity that exists within a tumor, as a BTIC has been shown to give rise to all the cellular subpopulations within a tumor [35, 40], including endothelial cells [41–44] but not immune cell infiltrates, which may arise from bone-marrow derived macrophages or brain-resident microglia [45]. Cancer may thus be thought of as a disease of unregulated self-renewal [6], as this property combined with the ability to assume a quiescent state to evade chemo and radiotherapy, together with enhanced DNA repair pathways, may allow BTICs to evade therapy. CD133, a known cell surface marker of BTICs, has been the focus of many studies as CD133+ populations not only initiated tumors *in vivo*, but are also known to be resistant to chemotherapy [46] and radiotherapy [47]. CD133+ cells maintain their radiotherapy-resistant

phenotype through the activation of DNA damage checkpoint pathway, allowing the cells to repair radiation induced DNA damage by arresting cell cycle [47]. Resistance to TMZ seen in CD133+ cells seems to be mediated through multiple mechanisms including higher expression of MGMT to maintain DNA repair mechanism and increased expression of anti-apoptotic genes and ABC transporters such as BCRP1 in CD133+ cell population [48]. The small molecule compound pyrrinium has been shown to inhibit self-renewal and eradicate the CD133+ GBM BTIC population that may persist throughout the course of treatment by generating a cellular hierarchy that contributes to ITH and the acquisition of drug resistance [49].

Although CD133 marks a more tumorigenic population in GBM, it does not mark the entire BTIC population in GBM as subsequent studies have shown that in some GBM samples, CD133-cells are also able to initiate tumors in xenograft models [50]. This led to the identification of additional markers of BTICs in GBM such as CD15 [51], integrin alpha6 [52] and L1CAM [53]. In addition, intracellular proteins such as RNA binding protein Musashi-1 [54], transcription factors Sox2 [55], Oct4 [56] and FoxG1 [57], and polycomb repressor Bmi1 [58, 59] that have a characterized functional role in driving normal neural stem cell self-renewal, have also been investigated as putative BTIC markers. Additional neurodevelopmental transcription factors such as Oct3, Sall2 and Olig2 have also been identified to play a role in GBM BTIC maintenance [56].

Future studies should now address whether BTICs are causative in tumor relapse and whether the same BTIC populations that drive tumor initiation also drive GBM recurrence.

Models to study intratumoral heterogeneity in GBM recurrence

Recent clonal evolution studies have relied largely on genome-wide sequencing alone, using the mutational profiles of bulk tumor populations to deduce the evolutionary trajectory followed by GBM through therapy. No studies have conclusively revealed how functional cell populations evolve through therapy in GBM to determine whether a pre-existing clone is driving therapy relapse in GBM, or therapy itself drives the emergence of a new population(s) that seeds the relapse. Studies so far have demonstrated that early somatic mutations in dominant clones drive tumor growth, whereas later mutations acquired during the course of treatment in heterogeneous low-frequency subclonal populations may aid in tumor recurrence and relapse. Current *in vitro* and *in vivo* models of GBM rely on primary tumor specimens at diagnosis to identify pathways that drive tumorigenesis, and extrapolate possible mechanisms of therapy resistance from the study of the treatment-naïve tumor specimen. However, these studies show that recurrent GBM is a divergent disease and therefore should be profiled in conjunction with the primary tumor to fully capture the evolutionary mechanisms driving therapy resistance and tumor relapse (Figure 1).

Although the CD133+ population has already been identified as both chemoresistant [48] and radioresistant [47], with recurrence having higher expression of CD133 [60], the combinatorial effect of TMZ and radiation on GBM BTIC populations has not been clearly studied to prospectively define whether these

treatments lead to selection of subclonal populations from which recurrence may arise. Animal models of GBM also fail to capture the progression of GBM from a primary treatment-naïve disease to a recurrent treatment-refractory disease. In fact, most genetically engineered mouse models of GBM have relied on mutations identified in a primary GBM patient cohort from studies by TCGA (animal models of GBM reviewed in [61]). Although genetically engineered mouse models have allowed researchers to explore the signaling pathways modulated by each mutation and how they impact tumor growth, studying each mutation in isolation prevents researchers from identifying the interdependence of multiple signaling pathways in GBM, their combinatorial role in disease progression and, most importantly, how the tumor will respond to therapy and escape treatment to seed relapse.

Patient-derived xenograft (PDX) models of GBM combat some shortcomings of genetically engineered mouse models by allowing the study of human GBM with its complete mutational profile in tumor initiation and disease progression. In fact, xenografts of GBM in immunodeficient mice have been shown to recapitulate the histopathological features of the parental GBM tumor, making PDX models a good surrogate for the study of GBM (Figure 2A) [35]. However, PDX models also lack validated protocols to study the progression of the disease through treatment and disease relapse.

To address the limitations of current *in vitro* and *in vivo* models of GBM, the focus must shift to the development of models that capture the evolving GBM population at tumor initiation and maintenance and, more importantly, through therapy and at recurrence (Figure 2B). Unlike previous studies that evaluated the independent effects of either chemotherapy or radiotherapy on GBM cells, Qazi et al. [62] used BTIC-enriched GBM cultures to characterize chemoradiotherapy-resistant cells. They developed and optimized a combined chemoradiotherapy protocol for *in vitro* GBM cultures based on clinically relevant doses of TMZ and radiation. Delivery of chemoradiotherapy to primary, treatment naïve GBM BTICs leads to increased expression of important stem cell genes (Bmi1 and Sox2), enriches for a CD15+ (a BTIC marker) population similar to that observed in patient-derived recurrent GBMs, and increases self-renewal capacity of the cells. In addition, gene expression profiles of *in vitro* chemoradiotherapy-treated GBM identified a previously unknown, hyper-aggressive subgroup of gliomas with significantly poor survival. This *in vitro* model captures aspects of recurrent GBM biology that would have been unidentified had the therapies been studied individually, and generates GBM recurrences in the laboratory, as patients with GBM recurrence are often palliative, disallowing repeat surgery for tissue sampling.

Extending these models to further delineate the role of ITH and subclonal selection upon recurrence in GBM, lentivector-mediated clonal tracking technology [63] can further delineate clonal dynamics of GBM recurrence. The concept of cellular heterogeneity is not cancer exclusive; rather normal cellular systems also display heterogeneity with the presence of multiple clonal subpopulations. A 2004 study by Mazurier et al. showed that within the normal hematopoietic system, the hematopoietic stem cell pool is highly functionally heterogeneous [64]. In the field of cancer research, cellular DNA barcoding technology can be used to answer pertinent tumor biology questions such as how the tumor evolves over the progression of the disease, how growth

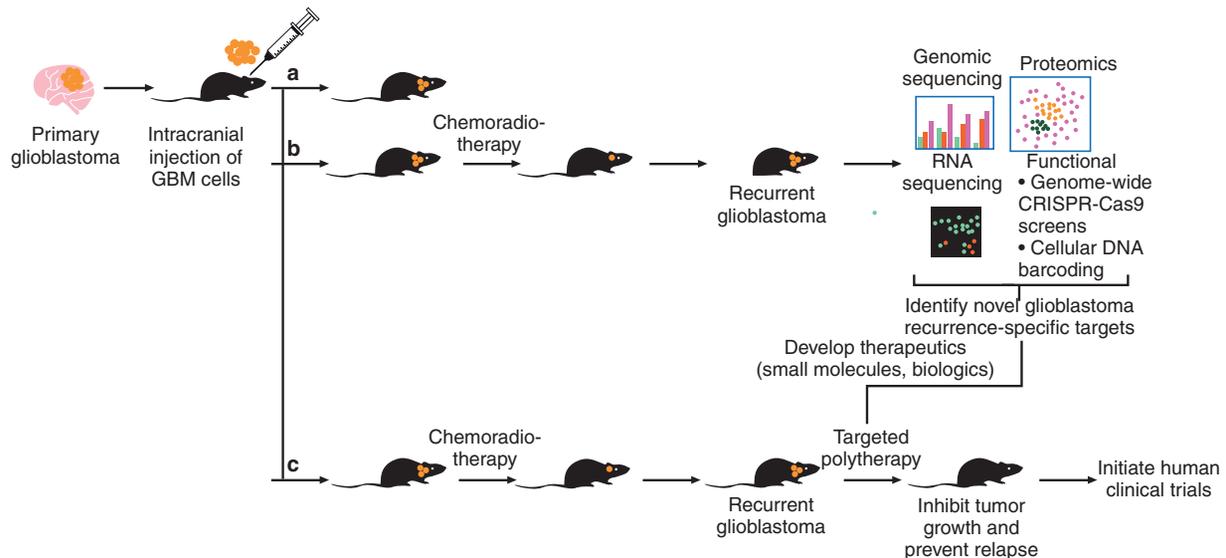


Figure 2. Development of recurrent glioblastoma models for the identification of novel targets to prevent disease relapse. Primary glioblastoma cells can be intracranially injected in mice to develop patient-derived xenograft models to study tumor biology. (A) The primary tumor engraftment is used to study the treatment-naïve glioblastoma. (B) Treatment of primary tumor with model-adapted chemoradiotherapy (radiation and temozolomide) similar to therapy administered to patients will lead to the development of recurrent glioblastoma, which can then be studied using multiple biological parameters (genomic, transcriptomic, proteomic, functional) for the identification of novel recurrence-specific targets. Therapies (small molecules and/or biologics) can then be developed for the recurrence-specific targets and can be tested in the xenograft model in a polytherapy approach (C) to characterize therapeutic potential and advance successful candidates to human clinical trials.

kinetics determine heterogeneity of tumor cells, and how tumor cells respond to therapy. To fully appreciate the complexity of a tumor population, studying tumor cells at single cell or clonal resolution is essential for the identification of drivers of tumor initiation, evolution and therapy resistance. Such studies have been undertaken in both leukemias and solid tumors (lung [65], breast [66] and colon [67]).

Although research has identified the presence of genomic heterogeneity in GBM and genetic subclones in primary and recurrent tumors, no studies have identified how clonal subpopulations present within GBM play a role in therapy resistance. Analysis of clonal dynamics in GBM following chemoradiotherapy will lead to the identification of clones that govern tumor recurrence, and will allow us to determine whether a pre-existing tumor clone or a divergent subclonal population that arises after therapy administration dominantly comprises recurrent GBMs. Use of such analyses will inform our understanding of the tumor biology of the primary GBM, and identify the pattern of recurrence in model systems to develop personalized therapeutics before the patient relapses.

The identification of all clonal subpopulations is indeed limited by our ability to perform multiple, sectional biopsies on patients with GBM. Multiple invasive brain surgeries pose risks for the patients as it may lead to further neurological complications. In addition, GBM is a highly invasive disease and despite total tumor resectioning, malignant cells might still be left behind in the patient's brain that can regenerate the tumor leading to relapse. However, recent technological advances are allowing researchers to explore and dissect GBM ITH through powerful new methods in validated model systems. With the advancement of genome wide CRISPR-Cas9 screening, identification of targets that are essential to recurrent GBM in maintaining tumorigenicity and therapy resistance will

pave new directions for the development of therapeutics for GBM. A recent study by Toledo et al. [68] identified PKMYT1, a protein kinase, as essential to BTICs for completion of mitosis and therefore a candidate therapeutic target for GBM. Another advancing technology that can be harnessed to understand GBM ITH is through the use of CyTOF (time-of-flight mass cytometry), which uses heavy metal tagged antibodies for highly multi-parametric single-cell proteomics [69]. Considering the heterogeneous landscape of GBM at the individual cell level, CyTOF lends itself to exploring the biological pathways governing multiple subpopulations of cells and identifying new markers for therapeutic targeting. The analysis of hundreds of proteins at single-cell level through a therapy model will lead to identification of key proteins and signaling pathways that underlie therapy resistance in GBM. Combining these technologies with single cell RNA sequencing [9] and phospho-proteomics [70] will only enrich the breadth of information acquired on GBM ITH and inform researchers on the complexity of cell signaling within the tumor, leading to the identification of key signaling nodes for therapeutic targeting (Figure 2B). Together these technologies can help researchers not only capture the ITH of GBM biology but perhaps also identify the 'Achilles' Heel' of GBM recurrence, which can then be targeted through empirically developed therapeutics.

The development of clinically relevant models of GBM recurrence combined with advanced techniques will afford the opportunity to identify novel targets specific to recurrent GBM (Figure 2C). Most current targets identified for therapeutics are derived from primary GBM specimens, despite the fact that recurrent GBM is a unique entity that is driven by biological programs distinct from its parent primary tumor. Models of recurrent GBM thus become paramount to bring efficacious therapeutics to the clinic in order to improve GBM patient prognosis.

Therapeutic implications of intratumoral heterogeneity

Taking into account the evolutionary dynamics of tumor populations, the therapeutic implications of ITH are of great importance for GBM therapy. The ongoing selection of cell populations through the course of disease development and particularly after the start of therapy suggests the need to study the evolving tumor biology throughout its disease course. The addition of new mutations and evolving tumor landscape as expected at recurrence in GBM would possibly require targeting of multiple clonal mutations in order to achieve prolonged therapeutic benefits (Figure 1). A primary limitation of the TCGA data is in its single biopsy study design, where the four subgroups gave an illusion of clonality. The clonal or subclonal nature of driver events would have to be clearly defined before targeted intervention by undertaking multiple tumor-sectional studies as well as developing models that recapitulate the underlying tumor biology that drives therapy resistance and recurrence in GBM. *In vivo* therapy-adapted models of GBM combined with new methodologies for the study of complex biological systems will allow researchers to explore this complex biology in a systematic way and begin to uncover novel targets with potential therapeutic benefits for patients with GBM recurrence (Figure 2).

Clinical trials in GBM with targeted therapies to date have failed to show significant improvement in patient survival. Myriad reasons can explain treatment failure in GBM, including inability to obtain a complete resection, challenges of drug delivery and crossing the blood brain barrier, limitations in clinical trial design and execution, and multidrug acquired resistance [71, 72]. RTK targeting has been a prime focus of clinical trials for GBM with EGFR, PDGFR and VEGF as prominent GBM specific targets. However, these trials have been confounded by the use of monotherapies against single RTKs (erlotinib for EGFR, imatinib for PDGFR and bevacizumab for VEGF), as efficacy of single agents is highly unlikely to succeed considering the complex and overlapping networks of RTKs with different driver RTKs in cellular subpopulations of GBM. In addition, therapy with single agents leads to selection of subclonal GBM populations, enriching for a therapy-resistant clone that then gives rise to recurrent GBM [70]. Targeting of EGFRvIII, a highly GBM-specific mutation, has also failed in trials, as the mutation has been shown to be present heterogeneously within the tumor population [73] and single targeting of EGFRvIII likely lead to the selection of wildtype EGFR-expressing populations that maintain tumor growth [74]. Immunotherapeutic approaches have recently gained momentum in the treatment of GBM as they promise better specificity and greater efficacy [75]. However, effective target identification for these therapies has been limited by the fact that large cohorts of genomic and transcriptomic studies have only included primary tumor specimens, with limited information on recurrent, treatment-refractory cell populations. To identify the converging and cooperative signaling pathways that maintain GBM growth through therapy and lead to recurrence, it will be critical to acquire large cohorts of datasets on recurrent GBM (genomic, transcriptomic and proteomic) and to combine these with experimental models to study GBM at subclonal levels through the use of multi-parametric technologies and/or single-cell analyses. The use of combining different modalities to treat GBM has been demonstrated to improve

progression-free survival (3 months) and overall survival (5 months) through the use of tumor-treating fields that disrupt cell division in combination with TMZ for newly diagnosed GBM patients [76]. Hence, polytherapeutic approaches that target multiple signaling pathways in recurrent GBM, along with multimodal therapy approaches would allow for the elimination of the most tumorigenic populations that drive treatment resistance.

Conclusion

GBM is a highly heterogeneous disease at the genetic, transcriptomic and functional level. Research within the past decade has shown the complex biology underlying GBM tumorigenesis and efforts have been made to characterize the disease further. Although initial studies by the TCGA were helpful in starting to dissect the immense heterogeneity found in GBM, it was soon realized that this heterogeneity is not only intertumoral but also intratumoral, with each tumor presenting a complex heterogeneous milieu of cell biology. ITH identified in GBM can in turn explain poor prognosis and inevitable tumor relapse. The resistance of GBM to current aggressive chemoradiotherapy can be attributed to the tumor's extensive cellular heterogeneity and the presence of multiple subclonal populations that invariably either respond to or escape therapy, regenerating treatment-refractory recurrent tumor. Current models for the study of GBM fail to directly address the problem of GBM recurrence and continue to focus efforts on understanding primary, treatment-naïve tumor biology. Clearly, new models of GBM must address both spatial and temporal ITH, and must broaden analysis beyond a single treatment-naïve sample at diagnosis to capture the evolution of recurrent, treatment-resistant disease. A detailed understanding of the evolutionary dynamics of tumor progression will provide insight into the associated molecular genetic mechanisms underlying GBM recurrence.

Models that incorporate chemoradiotherapy in the study of GBM will pave the path for a comprehensive understanding of GBM biology, pathways of therapy resistance and cell population dynamics in recurrence. The identification of pathways governing therapy resistance in clonal subpopulations will allow clinicians to offer patients therapeutics that selectively target the specific subclonal populations that drive GBM recurrence in each individual patient, leading to improved prognosis and outcomes.

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