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REVIEW

Intratumor heterogeneity, microenvironment, and mechanisms of drug resistance in glioma recurrence and evolution

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Abstract **Glioma is the most common lethal tumor of the human brain. The median survival of patients with primary World Health Organization grade IV glioma is only 14.6 months. The World Health Organization classification of tumors of the central nervous system categorized gliomas into lower-grade gliomas and glioblastomas. Unlike primary glioblastoma that usually develop *de novo* in the elderly, secondary glioblastoma enriched with an isocitrate dehydrogenase mutant typically progresses from lower-grade glioma within 5–10 years from the time of diagnosis. Based on various evolutional trajectories brought on by clonal and subclonal alterations, the evolution patterns of glioma vary according to different theories. Some important features distinguish the normal brain from other tissues, e.g., the composition of the microenvironment around the tumor cells, the presence of the blood-brain barrier, and others. The underlying mechanism of glioma recurrence and evolution patterns of glioma are different from those of other types of cancer. Several studies correlated tumor recurrence with tumor heterogeneity and the immune microenvironment. However, the detailed reasons for the progression and recurrence of glioma remain controversial. In this review, we introduce the different mechanisms involved in glioma progression, including tumor heterogeneity, the tumor microenvironment and drug resistance, and their pre-clinical implements in clinical trials. This review aimed to provide new insights into further clinical strategies for the treatment of patients with recurrent and secondary glioma.**

Keywords glioma; evolution mechanism; strategies; tumor heterogeneity; secondary glioma

Introduction

Glioma is the most common and aggressive brain cancer in adults and can be classified as grades I–IV based on histological features [1–3]. According to the clinical course, glioblastoma multiforme (GBM), which is referred to as grade IV glioma, can generally be classified into two subtypes [4]. Primary GBM refers to the vast majority of GBMs considered to form *de novo* in the elderly. Secondary GBMs (sGBMs) typically progress from lower-grade tumors and affect younger patients [5]. Recurrence of lower-grade glioma (LGG) and other tumors appears to be unavoidable despite considerable research performed in this field using various technologies in the last decades. Many extracellular tumor microenvironment (ETM) cell types are prevalent in brain tumors, but some important features that distinguish the normal brain from other tissues exist, including the composition of the ETM (e.g., microglia, astrocytes, and neurons), blood-brain barrier, and a previously "immune privileged" organs consideration in the human brain. Moreover, the skull provides a physical barrier to the swelling that often occurs following inflammatory reactions. Thus, interactions with the ETM require regulation within the brain. Hence, the mechanism underlying glioma recurrence and the evolution patterns of glioma remain controversial.

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Studies have revealed individual and spatiotemporal evolution patterns of the primary GBM genome [6,7] and have identified key genomic events and subgroup alterations during the progression of glioma [8–11]. Few researchers have systematically reviewed the mechanisms and patterns that occur in the progression and recurrence of glioma [12]. Moreover, few reviews have discussed the differences in intratumor heterogeneity (ITH), microenvironment, and differences in the mechanisms underlying drug resistance between glioma and other types of tumors.

We reviewed the currently available literature on the mechanisms of glioma progression, including tumor heterogeneity, ETM, and drug resistance. We described some evolutional patterns during the development and progression of glioma, to provide new insights into further strategies for the treatment of patients with recurrent or secondary glioma.

Gradualism and punctuated evolution of cancer

Approximately half of the mutations, e.g., driver mutations in tumor protein p53 (TP53), BRAF, and ATRX, identified in the initial tumor of LGG are no longer detectable at the time of recurrence. This phenomenon indicates that recurrent tumors are often seeded from heterogenetic cells derived from ancestors at early stages [9,11]. The long-standing debate on the evolution of cancer has focused on whether tumors evolve gradually through the sequential accumulation of alterations during clonal expansions or are characterized by punctuated progression [13]. The gradual evolution hypothesis is supported by the presence of clock-like alteration signatures observed in patients [14]. Different patterns of cancer evolution have been described in various cancer types. Evidence for the selection of driver events in cancer development and therapeutic pressure is currently limited. Therefore, ITH in the development of colorectal carcinoma can follow the laws of neutral growth after a "big bang" of diversity early events in its evolution [15]. Another evolution pattern known as punctuated or parallel evolution is described as an independent pattern of similar evolution characterization initiating from a single ancestral clone [16]. Clone cells harboring mutations did not expand, despite the proliferative phenotype typically conferred by this mutation in the recurrence of other cancer types [17,18]. This finding indicated the ineffectiveness of sequential monotherapy.

Similarly, glioma contains various heterogenetic tumor clones and evolutionary patterns. Multiregional biopsy and spatiotemporal genomic architectural study may provide information regarding the clonal evolution from initial glioma to recurrence [11,13,19]. Samples from the same tumor mass were determined to have shared genomic and expression patterns using bulk and single-cell data of 127 samples obtained from 53 patients. In contrast, multiregional, separately localized tumors, or long-term recurrent GBMs are seeded from different clones at an early stage [19]. Strikingly, more complex clonal evolution was found in recurrent GBM. Alternative models for the branching pattern are also proposed, in which the recurrent samples are seeded from a lineage nested within the tumor, perhaps selected by therapy [11,19]. Model I could be described as a clonal mutation in the initial sample and absent in recurrence, whereas model II could be described as a subclonal mutation in the initial sample and clonal in recurrence (Fig. 1A). Bao *et al*. showed that the PTPRZ1-MET (ZM) fusion was enriched in patients with sGBM [20] and subsequently found that MET exon 14 skipping (METex14) accompanied by ZM fusion promoted malignant phenotypes of glioma. The subclonal level of METex14 in initial tumors was associated with poorer overall survival than that reported in *MET*ex14-negative patients [21], indicating the model II evolution pattern in sGBM patients with MET alterations. A chemical screening of patient-derived glioma cells showed that therapeutic response was associated with genetic similarity [19]. Owing to the presence of the linear evolution mode in these tumor cells, local recurrent tumors respond well to targeted treatment. However, multifocal GBMs develop through parallel evolution [22]. The branching evolution pattern and estimates of evolutionary rate suggest that the relapse-related clones typically existed years before the diagnosis [11]. The recent glioma evolutional study "GLASS" demonstrated that the clonal architecture of each tumor remained similar, whereas the presence of subclonal selection was associated with the clinical outcome [12]. The evolution pattern in the landscape of driver clones was correlated with the distant appearance of a recurrent tumor from the initial tumor [7]. This phenomenon indicated the misleading therapy involving the targeting of the genomic profile of the initial tumor for the distally recurring tumors.

Various pathways are involved in the evolution of GBM. Genetic alterations of the p53 pathway were identified as primary molecular markers with a high number of subclonal alterations in GBM [6]. Among recurrent GBMs, 11% of tumors harbor mutations in latent transforming growth factor β binding protein 4 (LTBP4), which encodes a protein that binds to the transforming growth factor β (TGF-β). Inhibition of LTBP4 in GBM cells restrains the TGF-β pathway and decreases cell proliferation, highlighting the function of this pathway as a potential predictive marker in GBM [11]. Other genetic alterations, including ETS variant transcription factor 1 (*ETV1*), cyclin dependent kinase 6 (*CDK6*), NF-κB (complex), interleukin 1B (*IL1B*), *IL6*, *AKT*, and vascular endothelial growth factor (*VEGF*) were identified as candidate genes and potential signaling regulators of

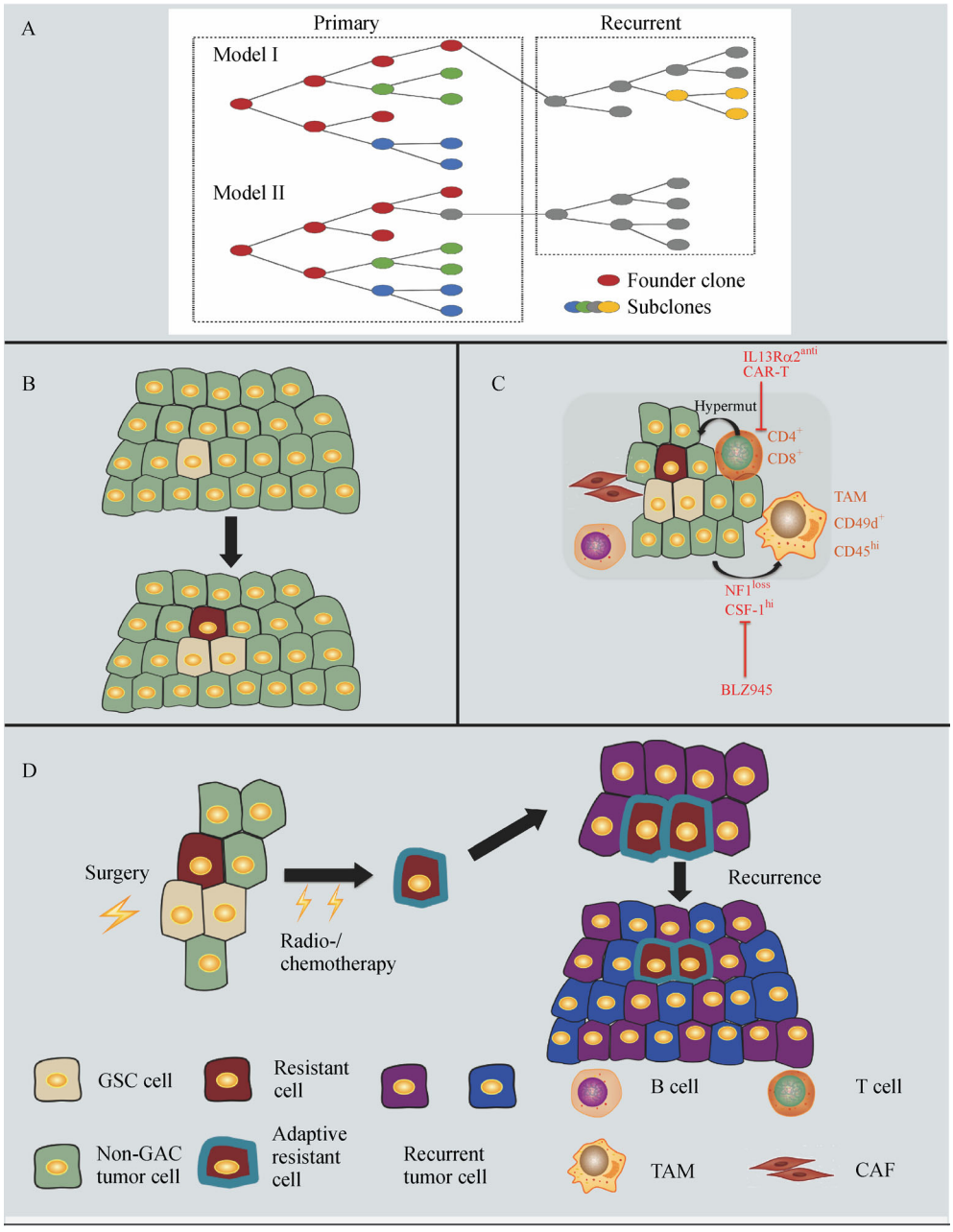

Fig. 1 Mechanisms of glioma progression. (A) Patterns of glioma evolution. Model I: both samples are monophyletic ("branching evolution") due to founder clonal genetic alterations. Model II: recurrence monophyletic, nested within tumors owing to the subclonal genetic alterations. Red circles indicate the founder clone, whereas multicolor circles indicate subclones. Circles indicate the time point of diagnosis of primary and recurrent glioma. (B) Heterogeneity in glioma. (C) Microenvironment of glioma. (D) Drug resistance in glioma. All cell types are listed at the bottom of the figure.

chromosome gain or loss in recurrent GBM [23]. These findings are consistent with those generally observed in GBM samples in comparison with normal brain tissue.

Mazor *et al*. investigated the function of isocitrate dehydrogenase (*IDH*) mutation from LGG in recurrence and discovered that deletion of *IDH1* was followed by clonal evolution and recurrence at a higher grade. Deletion of *IDH1* leads to decreased concentration of 2-hydroxyglutarate, maintenance of the glioma CpG island methylator phenotype, and DNA methylation reprogramming outside CGI. This finding indicates that in some patients, mutant *IDH1* and 2-hydroxyglutarate are not required for recurrence despite the initiation of gliomagenesis through the mutation of *IDH1* [24]. Interestingly, genes transcriptionally dysregulated through promoter methylation and enriched in cell cycle pathways were associated with malignant progression to high-grade gliomas [24].

Mechanisms of glioma recurrence and malignant progression

Hanahan and Weinberg reviewed the different ways of activating cancer invasion and metastasis [25], including the epithelial-mesenchymal transition program, heterotypic contributions of stromal cells, plasticity, and the daunting complexity of metastatic colonization. In glioma, *PTEN* and *p53* alterations [26], *IDH* [27] and *ATRX* [28] mutations, and telomerase reverse transcriptase promoter mutation [29] lead to the initiation of glioma and impact the overall survival time. However, the underlying mechanism of these alterations and other factors involved in glioma progression and recurrence remain unknown. ITH with genomic instability and hyper-mutation, the ETM (consisting of immune inflammatory cells, cancer stem cells (CSCs), endothelial cells, pericytes, cancer-associated fibroblasts (CAFs), and progenitor cells of the tumor stroma), and metabolic deregulation have recently been found as enabling and emerging characteristics of cancer pathogenesis and neoplastic proliferation.

ITH

ITH has been identified since the 1800s [30]. It is recognized as a key reason for therapeutic failure, drug resistance, and tumor progression [31,32]. However, ITH had not been distinctly elucidated until the development of advanced technology, such as next-generation sequencing. Through this approach (occasionally single-cell sequencing), studies demonstrated that distinct subpopulations cooperate to promote tumor maintenance, growth, and progression [33–36].

A recent study regarding LGG [9,24], GBM [7,37], and other tumor types [23,38] demonstrated the presence of different subclonal alterations between initial and recurrent tumors. The biological process and treatment implications of ITH have been recently reviewed. Divergent extents of ITH have been found in brain tumors, including diffuse intrinsic neuroblastoma [39], pontine glioma [40], LGG [9], and GBM [7,11]. The characteristics of heterogeneity in GBM have recently been identified. As implied by the term "multiforme," ITH at a high level exists in this type of brain tumor [41]. Primary GBM samples showed strong association with classical and mesenchymal subtypes, as shown by RNA sequencing, thereby confirming the heterogeneity in GBM [22]. Analysis of copy number profiles revealed a high degree of genetic instability among different tumor cells with a high level of heterogeneity. This phenomenon indicated that genomic instability and ITH increase as GBM cells increase in tumorigenicity [23]. ITH is relatively well recognized in GBM [42]. However, fluorescence *in situ* hybridization [43], multi-region sequencing [9,44], and single-cell sequencing [45,46] revealed a more detailed characterization of LGG heterogeneity. *IDH1* mutation occurs early in the course of LGG followed by deletion or amplification after tumor progression and clonal expansion at a higher grade [10]. As genome doubling and ongoing dynamic chromosomal instability are associated with ITH as an early event [47], further investigation should be performed regarding the correlation between ITH and IDH mutation. DNA methylation loss is identified during progression from LGG to high-grade glioma because of heterogeneity of the initial tumor [24]. However, ITH could not explain the majority of genetic alterations between the initial and progression tumors [9]. Geographic heterogeneity of the tumor is not responsible for the genomic divergence in distant recurrence samples [24]. Furthermore, intraGBM heterogeneity does not explain the large number of alterations uniquely detected in initial and recurrent tumors [6].

Future molecular targeted therapies should focus on at least four types of cancer cells based on their different properties and response to treatment, namely, primary glioma stem cells (GSCs), recurrence-initiating stem-like cancer cells, proliferating cells, and non-GSCs, as suggested by some researchers [48]. Inhibitors of the Wnt, sonic hedgehog signaling molecule (SHH), and Notch pathway are good candidate therapies for GSCs [8,49]. Targeting of adaptive resistance mechanisms and blocking of immune suppression can be accomplished by eliminating the populations of recurrence-initiating stem-like cancer cells [6,50], as shown in Fig. 1B.

ETM influences tumor progression

Multiple extracellular stromal cells converge to support the tumorigenic process by sustaining cell growth, invasion, and metastasis, leading to the following: inhibition of B and T cell responses, the recruitment of tumor-associated macrophages (TAMs), and the influence of CAFs. Unlike tumor cells, stromal cells within the ETM are genetically stable and thus become potential therapeutic targets with reduced risk of resistance and tumor relapse [51]. Interestingly, initial tumors with diverse recurrent potentials differ in their composition of both tumor- and stroma-derived ETM components [52]. Furthermore, the composition of the ETM is associated with clinical prognosis.

Another predominant factor in ETM associated with tumor progression is angiogenesis, which initiated a paradigm shift in cancer evolution [53,54]. Tumor vascularization requires the mutual interaction among multiple ETM cell types, such as TAMs, mesenchymal stem cells, and CAFs, whose phenotype is often regulated by hypoxia [55–57], as shown in Fig. 1C. Studies reported the effect of CAFs on the migration of glioma cells through their angiogenesis activity [58] or similar functional

properties between CAFs and GBM stromal cells [59]. The role of CAFs in the malignant development of glioma needs further investigation.

In glioma, ETM is correlated with transcriptomic subtype multiplicity and ITH, which are involved in glioma cell clone expansion. Macrophages/microglia, $CD4^+$ T lymphocytes, and neutrophils have been identified in the glioma microenvironment through *in silico* cell sorting. Deficiency of the neurofibromin 1 (*NF1*) gene leads to increased TAM infiltration and reveals a decrease in invading monocytes with a subtype-dependent increase in macrophages cells at recurrence. $CD8^+$ T cell enrichment was further associated with hypermutation at diagnosis or recurrence [60]. These findings demonstrate that the microenvironment can normalize intrinsic tumor cells and re-regulate stromal cells. Therefore, targeting of the microenvironment rather than targeted ablation of tumor cells may be a more effective option for cancer treatment.

Further emerging examples of ETM-directed therapies neutralize tumor-associated chronic inflammation rather than targeting microenvironment cells. A colony stimulating factor 1 receptor (CSF-1R) inhibitor was recently used to target macrophages and microglia in the ETM of gliomas [61]. A patient with recurrent multifocal GBM received chimeric antigen receptor–engineered T cells targeting the tumor-associated antigen interleukin-13 receptor α 2 (IL13Rα2). Following chimeric antigen receptor T cell therapy, a decrease in all intracranial and spinal tumors was observed along with symptom relief and prolonged survival time [62].

Drug resistance and tumor relapse

Heterogeneity is associated with endogenous drug resistance in tumors. Heterogeneity-induced resistance can arise through two main mechanisms, namely, (1) pre-existing therapy-resistant clones prior to treatment and (2) *de novo* alterations acquired after treatment. Even with the most effective treatment, most patients exhibit incomplete drug response. Moreover, the residual tumor bulk commonly contains a small population of quiescent drug-resistant clones that survive the therapy due to alternative metabolic and epigenetic pathways [63–67]. Acquired drug resistance is often attributed to the selective expansion of pre-existing therapy-resistant subclones. However, some preclinical studies revealed *de novo* alterations of resistance alterations, which lead to the evolution of drug-tolerant cells [68,69]. Although these resistance alterations are often referred to as "acquired," several studies identified *de novo* resistance alterations that are present at low frequencies in pretreated tumor tissues [70,71]. Bulk and single-cell sequencing demonstrated acquired malignant phenotypes after targeted therapy, including enhanced mesenchymal and growth factor signaling and decreased antigen presentation pathway, which may enable immune check-point avoidance. Some of the pre-existing subclones in pretreatment specimens with these phenotypes become dominant after chemotherapy, indicating the selection pressure for these resistance phenotypes [72].

Stem cells are responsible for the endogenous inducement of tumor progression. These cells are undifferentiated biological cells, which is a definition that is based on their capacity for long-term self-renewal to differentiate into multiple cell lineages. In numerous adult tumor tissues, stem cells (referred to as CSCs) are responsible for tumor homeostasis and regeneration [73]. CSC populations contribute to the occurrence of drug resistance through a variety of mechanisms, including self-renewal, quiescence maintenance, survival ability, and drug efflux [74]. Each category reflects specific CSC characteristics and provides anti-CSC strategies in the treatment of advanced cancers. In tumors constructed with CSCs and non-CSCs, the sensitivity of CSCs to chemotherapy inhibits tumor growth regardless of the remaining non-CSCs, inducing tumor regression. In case of non-CSC elimination, CSCs sustain tumor growth, inducing tumor relapse. The capacities to maintain tumor propagation, induce inherent resistance to clinical therapy, and contribute to tumor progression are fundamental properties of CSCs. Stem cells are located within the subventricular zone and express stem cell marker nestin in normal brain [75]. GSCs are identified in glioma tissue. Treatment with the standard drug temozolomide (TMZ) preferentially targets rapidly cycling tumor cells, whereas nestin-positive CSCs re-enter and retard the cell cycle after TMZ administration, thereby contributing to tumor relapse [76]. Resistant CSCs can be either intrinsically resistant to therapy (e.g., resistance to DNA damage and expression of multidrug resistance) or extrinsically instructed by the ETM (e.g., immune evasion, autophagy, and TAMs). Researchers demonstrated enriched CSCs following chemotherapy or radiotherapy, indicating the therapy-induced selection of cancer cells with CSC properties. Radiation induces high expression of $CD133^+$ CSCs in GBM xenografts [77]. Epithelial-mesenchymal transition and CSC properties have been identified in GBM cell lines that acquired resistance to the anti-VEGFA bevacizumab [78]. In GBM, lineage-sorting experiments suggested that TMZ resistance is accompanied by the expansion of the CSC subclone [79]. Accordingly, targeting CSCs/GSCs necessitates a comprehensive elucidation of the mechanisms that lead to resistance to radiotherapy or chemotherapy.

Exogenous tumor resistance is associated with hypoxia and the ETM. Hypoxic cancer cells prevent the destabilization of the strands of DNA by ionizing radiation, indicating that molecular adaptations induced by oxygen-sensitive mechanisms impact responses to radiotherapy

and tumor recurrence [80]. Homozygous mutation of Kelch-like ECH associated protein 1/TP53 (KEAP1/TP53) promotes airway basal stem cell self-renewal, leading to expansion of mutant stem cell clones. Meanwhile, deletion of KEAP1 increases tumor resistance to oxidative stress and radiotherapy [81]. This finding suggests that genomic mutations promote tumor progression, metastasis, and resistance to therapy through mutual effects between tumor and hypoxia in the microenvironment (Fig. 1D).

Models for the progression of glioma following radiotherapy or chemotherapy identified two patterns according to the presence of residue cell clones [6]. In the ancestral cell origin model, all dominant glioma clones from the primary tumor (unlike refractory cells) are removed after therapeutic intervention. The ancestral glioma cell accumulates new alterations that are identified in the recurrent tumor. In the clonal evolution model, radiotherapy or chemotherapy removed most of the initial tumor clones, but cells from the initial glioma clones survived and proliferated to a recurrent tumor. Endogenous pathways are altered after glioma progression. After treatment with TMZ, the refractory glioma is characterized by hypermutation and dysregulation of the retinoblastoma and AKT-mammalian target of rapamycin (AKT-mTOR) pathways [9]. In contrast to *IDH1*-mutated gliomas, *IDH1*-wild-type primary GBMs rarely present hypermutation after TMZ chemotherapy, demonstrating a low risk for TMZ-induced hypermutation in patients with GBM who received standard treatment [7]. Only 15% of GBM cases harbor hypermutation in highly expressed genes at recurrence [11]. The metabolic status of highly proliferative stem cells and cancer cells is similar [82,83]. Menendez *et al*. found that metabolic reprogramming in gliomas, which is influenced by the ETM, contributes to drug resistance and tumor relapse. Solute carrier family 2 member 3 (SLC2A3; previously termed GLUT3) is highly expressed in GSCs and indicates resistance to radiotherapy or chemotherapy [84]. Pyruvate dehydrogenase kinase 1 (PDK1) inhibitor dichloroacetate enhanced the activity of pyruvate dehydrogenase in rat GSCs and increased the sensitivity of cells to chemotherapy and radiotherapy *in vitro* [85].

Summary and perspectives

The mechanism underlying glioma evolution and the dynamic interaction between glioma cells and the ETM have been studied by many scientists [12,86,87]. However, the field of therapeutics targeting the evolution of glioma and the prevention of glioma progression is currently in its infancy (Table 1). The resulting genetic instability of glioma results in subsequent heterogeneity, which is maintained by treatment with TMZ or other targeted therapeutic selective pressures. Identification of clonal dynamics in glioma samples obtained from sites of resistance may lead to the development of further treatment options that address tumor heterogeneity. However, this approach should ideally be combined with the use of noninvasive liquid biopsy sampling, including CSF of patients with glioma, which enables easier surveillance. The clinical implications of relapse subclones need to be examined in CSF samples for the early diagnosis of recurrent glioma [72]. Technological and computational advances for exploring the glioma genome and its ETM will offer a deeper understanding of the evolutionary trajectories of this disease. On the basis of our current understanding of the evolution patterns of glioma, the use of combination therapies (e.g., combinations of targeted and immune therapies) may be the most promising approach. Clinical practice including clinical trials should also be dynamic with the timely adjustment of antitumor strategies. Although numerous clinical trials have investigated recurrent glioma, a deeper understanding of the mechanisms and identification of additional targets of glioma recurrence are warranted in this setting.

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Table 1 Completed clinical trials for recurrent glioma

NCT number	Title	Conditions	Interventions
NCT00271609	Bevacizumab for recurrent malignant glioma	Recurrent high-grade gliomas/malignant gliomas	Drug: bevacizumab
NCT01738646	Ph II SAHA and bevacizumab for recurrent malignant glioma patients	Recurrent glioblastoma multiforme/malignant glioma/adult brain tumor	Drug: vorinostat/drug: bevacizumab
NCT00619112	Temozolomide in treating patients with recurrent high-grade glioma	Recurrent central nervous system neoplasm	Drug: temozolomide
NCT00597493	Ph. 2 sorafenib + protracted temozolomide in recurrent GBM	Recurrent glioblastoma multiforme	Drug: sorafenib and temozolomide
NCT00575146	Ketogenic diet for recurrent glioblastoma	Recurrent glioblastoma	Dietary supplement: TAVARLIN
NCT00777153	Cediranib in combination with lomustine chemotherapy in recurrent glioblastoma	Recurrent Glioblastoma	Drug: cediranib/drug: lomustine chemotherapy/drug: placebo cediranib

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Compliance with ethics guidelines

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