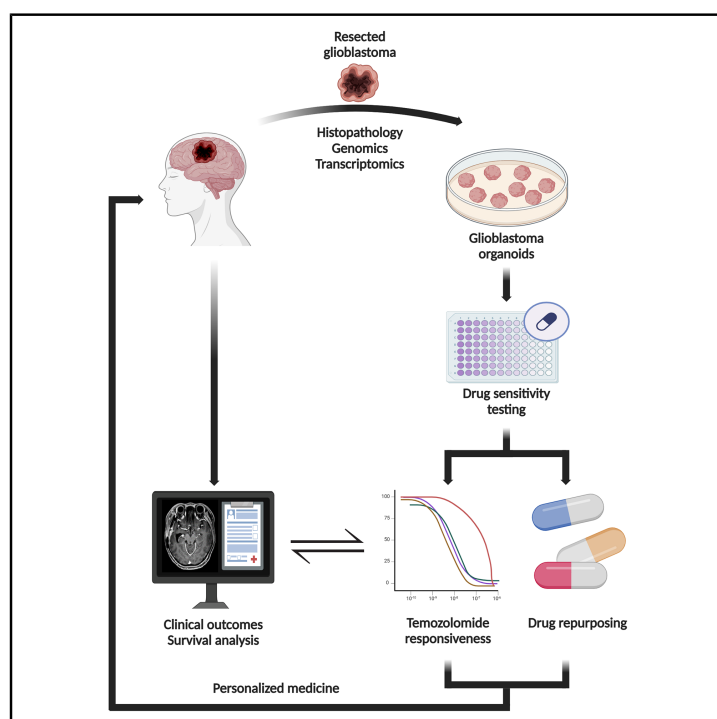


Patient-derived organoids predict personalized drug response and reveal alternative therapeutic options in glioblastoma

Graphical abstract



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In brief

Park et al. show that patient-derived glioblastoma organoids faithfully recapitulate parental tumor features and predict clinical responses to temozolomide more accurately than current biomarkers, including MGMT status. This platform reveals heterogeneous resistance mechanisms and enables personalized drug screening, identifying lazertinib as a promising therapeutic option for temozolomide-resistant glioblastoma.

Highlights

- Glioblastoma organoid (GBO) recapitulates histopathological and genomic features
- GBO outperforms MGMT status in predicting temozolomide response
- GBO reveals drug resistance mechanisms and identifies drug candidates
- *In vivo* GBO model validates lazertinib as a therapeutic option for resistant tumors

Article

Patient-derived organoids predict personalized drug response and reveal alternative therapeutic options in glioblastoma

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SUMMARY

Glioblastoma remains a highly aggressive brain tumor with limited treatment options and poor prognosis. Temozolomide (TMZ) is the only approved first-line therapy, but frequent resistance limits its efficacy, highlighting the urgent need for alternative treatments. We establish 18 glioblastoma organoid (GBO) lines that preserve the histopathological and genomic features of the parental tumors, enabling comparison with clinical outcomes. GBO-based drug sensitivity testing (GBO-DST) showed a strong correlation with progression-free survival and outperformed O⁶-methylguanine-DNA methyltransferase (MGMT) methylation status in predicting TMZ response. Transcriptomic analysis reveals mechanisms of TMZ resistance, including mismatch repair deficiency, elevated MGMT expression, and upregulation of genes associated with axonogenesis and cell adhesion. Using GBO-DST, FDA-approved drug screening identifies regorafenib and lazertinib as effective treatment alternatives. Additionally, lazertinib demonstrated superior efficacy to standard therapies in GBO transplantation mouse model. These findings underscore the potential of GBO-DST as a robust platform for precision oncology in glioblastoma.

INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive form of malignant primary brain tumor in adults and accounts for more than half of all malignant brain tumors. In the United States, the average annual age-adjusted incidence rate of GBM is 3.19 per 100,000 persons.¹ The current standard treatment for GBM, based on a landmark study published in 2005, consists

of maximum surgical resection followed by temozolomide (TMZ)-based concurrent chemoradiation therapy (CCRT) and 6 cycles of maintenance TMZ.² Despite recent advances in multimodality therapy for GBM, including surgery, radiation therapy, systemic therapy, and locoregional therapy, the overall prognosis remains less than 2 years, and tumor progression is inevitable.³ Over the past 30 years, nearly all phase 3 trials, including those involving targeted therapies and immunotherapies, have

failed to demonstrate survival benefits in GBM.^{4–7} Systemic therapy options to prolong survival are limited beyond TMZ, and no established standard of care exists, particularly for recurrent GBM. The latest National Comprehensive Cancer Network guideline recommends active enrollment in clinical trials for patients with GBM.⁸ Meanwhile, the fifth edition of the World Health Organization Classification of Tumors of the Central Nervous System (CNS), the international standard for classifying brain and spinal cord tumors, emphasizes the essential role of molecular diagnosis in GBM.⁹ However, reliable assays or molecular biomarkers for predicting prognosis or treatment responsiveness remain scarce, aside from O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation, which confers a favorable prognosis and response to alkylating agents, including TMZ.¹⁰ There is an ongoing need for the discovery of effective therapeutic drugs and the development of platforms capable of predicting personalized treatment responses in patients with GBM.

Various preclinical models have been utilized to evaluate tumor responses to chemotherapeutic agents. While traditional two-dimensional cultures of immortalized cell lines are commonly used, they are unable to replicate critical features of primary tumors, such as stemness, genetic heterogeneity, and the tumor microenvironment (TME).¹¹ Animal models, including patient-derived xenografts (PDXs), preserve the structural and genetic characteristics of the tumor and simulate the physiological and pathological processes within the TME.^{12,13} However, animal models are costly, time consuming, and limited in their ability to mimic human disease due to interspecies differences. Patient-derived organoids (PDOs) have emerged as the most promising tool for closely mimicking human diseases while overcoming several limitations of other models.^{11,14} A growing body of evidence indicates that PDOs cultured from a series of solid tumors, including colorectal, lung, ovarian, breast, liver, esophageal, and other cancers, successfully replicate the genotypic and phenotypic characteristics of the original specimens.^{15–24} Furthermore, recent studies have shown that PDOs accurately reflect treatment response outcomes of chemotherapeutic agents, radiation therapy, and targeted therapy, exhibiting a strong correlation with actual clinical responses.^{22,23,25}

Since the landmark studies by Yoshiki Sasai group and Jurgen Knoblich group, brain organoid models have been increasingly used to study brain diseases, including GBM.^{26,27} Hubert et al. successfully cultured patient-derived GBM as complex organoids composed of multiple cell components using Matrigel-based three-dimensional (3D) culture methods.²⁸ Subsequently, Jacob et al. coined the term “glioblastoma organoid” (GBO) and refined the protocol to rapidly generate GBOs in a defined, serum-free medium without EGF/bFGF or extracellular matrix components.²⁹ They showed that GBOs maintained molecular features and cellular heterogeneity of parental GBMs and reproduced phenotypic properties *in vivo* with PDXs. Furthermore, GBOs were employed to test responses to TMZ as well as targeted treatments and chimeric antigen receptor T cell immunotherapy.²⁹ Several authors have also highlighted the potential of GBOs as a platform for assessing clinical responses to diverse drugs and facilitating personalized medicine.^{30–32} However, GBO-based drug sensitivity testing (GBO-DST) for predicting

clinical drug responses requires extensive validation within a well-structured clinical cohort. Furthermore, in-depth genomic and transcriptomic analyses of GBO-DST results are necessary.

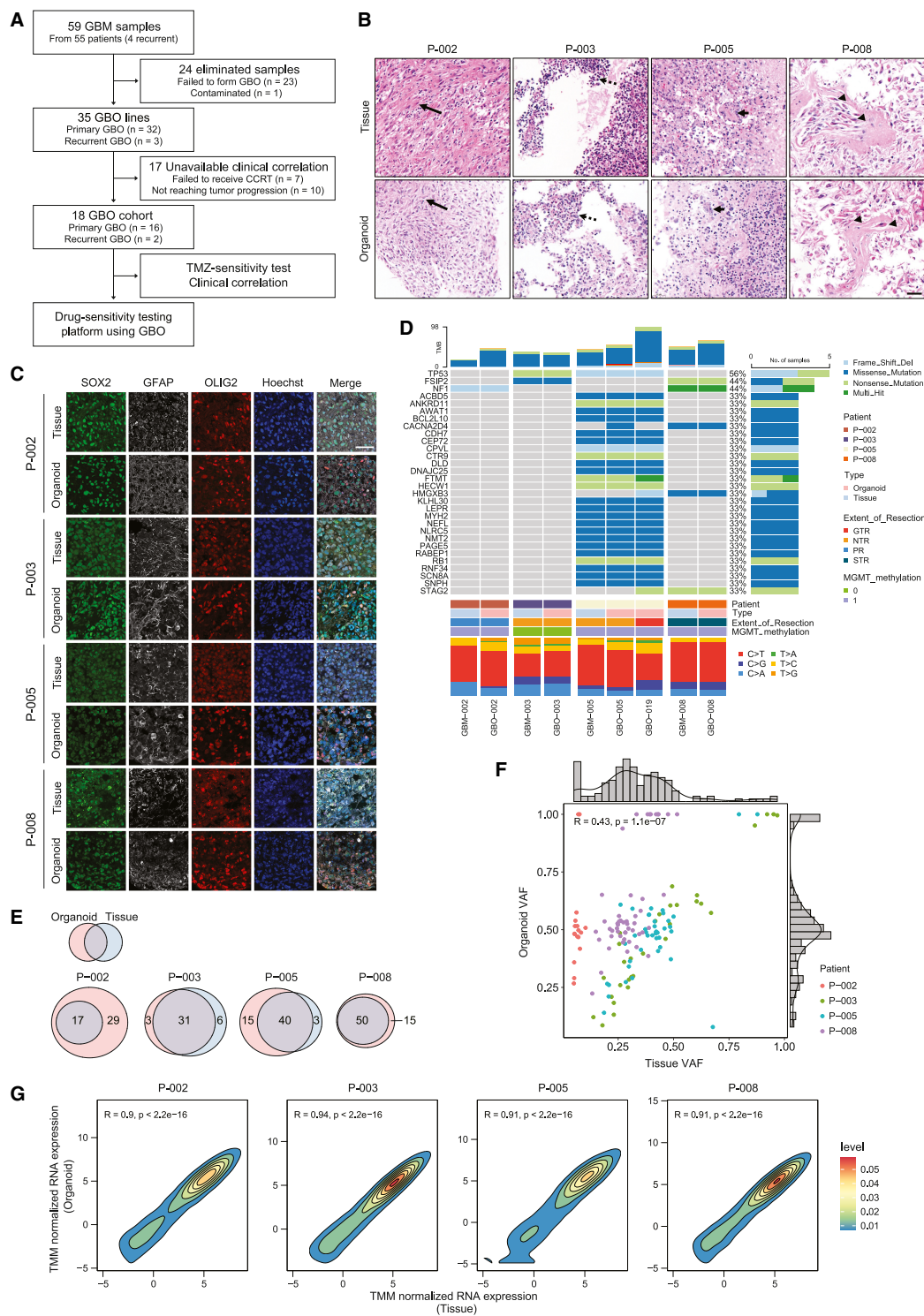
Here, we establish a GBO-based pipeline that enables efficient drug response testing and the identification of potential therapeutics. Our findings reveal that GBOs can more accurately predict individual patient responses to TMZ compared to traditional biomarkers, such as MGMT methylation status. This underscores the GBO platform’s promise as a more reliable tool for tailoring treatment strategies, with the potential to improve clinical outcomes for GBM patients. In addition, we tested four FDA-approved cancer drugs and identified lazertinib, a third-generation epidermal growth factor (EGFR) inhibitor, as an effective candidate for suppressing the growth and survival of GBOs. Furthermore, we validated the efficacy of lazertinib in an *in vivo* GBO transplantation model against standard therapies, demonstrating its potential as an alternative therapeutic option to advance personalized medicine for GBM. Our findings highlight the pivotal role of the patient-derived GBO platform in driving personalized treatment strategies and accelerating drug screening for GBM, with the potential to expedite its clinical implementation.

RESULTS

Patient-derived GBO recapitulates histopathologic features of original GBM

In our study, 59 GBM samples (including 4 recurrent samples) for the generation of GBOs were derived from 55 patients. Fresh tumor samples were transferred to the laboratory immediately after surgery, and GBOs were cultured following an established protocol.³³ Based on previous studies, we routinely dissected GBOs when their diameters exceeded 1 mm to prevent necrotic core formation. After dissection, the GBOs maintained their spherical morphology and increased in size (Figure S1A). While the growth rates varied among GBOs, all consistently increased in size over time (Figure S1B). The variability is likely due to the heterogeneity of the patient-derived tissues, reflecting differences in gene expression profiles and cellular microenvironments. Despite this variability, all GBOs demonstrated robust growth, confirming the viability of the culture system, indicating that our GBO system was well-established and comparable to those reported by other groups.^{29,33} Standardizing the growth rates in future studies will be essential to improve the evaluation and interpretability of experimental outcomes.

A total of 36 GBO lines were successfully established, with a success rate of 61.0%. After excluding one contaminated line (GBO-009), 35 GBO lines were maintained and stored in the biobank (Table S1). An evaluation of TMZ responsiveness in the clinical setting was not possible for 17 patients: 7 were unable to receive TMZ-based adjuvant therapy due to poor performance status and 10 had not yet reached the primary endpoints of tumor progression and/or death at the time of analysis. Consequently, 18 GBOs, including 16 primary and 2 recurrent GBOs, were included in the in-depth analysis (Figure 1A). To investigate the causes of failure in GBO culture, univariate and multivariate risk factor analyses were conducted using key clinical and tumor



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characteristics (Table S2). Extensive infiltration, multifocal enhancement, and/or leptomeningeal disease on initial imaging, as well as lower Ki-67, were identified as significant factors associated with impaired organoid formation. In contrast, age, sex, and MGMT methylation status were not.

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) were conducted to evaluate whether GBOs retained the histopathologic features of their original GBM tissues. H&E staining revealed a remarkable similarity between GBOs and GBM tissues in terms of cell composition and morphology (Figure 1B). For instance, GBO-002 exhibited spindle cells and abundant cytoplasm, closely resembling the parental tumor (solid arrow). Both GBO-003 and its parental tumor showed abundant small cells (dashed arrow), while GBO-005 and GBM-005 shared the presence of giant cells with abundant cytoplasm and small cells (small arrowhead). In addition, GBO-008 showed polygonal or bipolar cells like its parental tumor (larger arrowhead). To further characterize cellular identities, IHC analyses were conducted using a panel of neurodevelopmental cell type markers, including GFAP (astrocyte-like), SOX2 (neural progenitor and glioma stem cell-like), and OLIG2 (glioma stem cell and oligodendrocyte progenitor-like). When comparing the original GBM tissues and GBOs, all markers were robustly expressed, demonstrating significant concordance in neurodevelopmental cell composition (Figure 1C). In summary, these findings demonstrate that the morphology and biomarkers of the original tissues are preserved in GBOs, suggesting that GBOs successfully recapitulate the histopathologic characteristics of their respective GBMs.

GBOs maintain the genetic landscape of parental tumors

We utilized whole-exome sequencing (WES) to assess whether GBOs faithfully replicate the genomic profile of their parental tumors. Five GBOs, including one recurrent GBO, and their corresponding tumor tissues with available blood samples were selected for somatic mutation profiling to exclude germline variants. We characterized the genomic landscape of the GBOs and their parental tumors, including somatic mutations such as single-nucleotide variants and small indels, as well as copy number variants (CNVs).

We identified 445 somatic mutations in 265 genes from 9 samples. Somatic mutations in the most frequently mutated top 30 genes are shown in Figure 1D. In GBM tissues, all somatic mutations that occurred in the top 30 genes were also identically observed in paired GBOs. The Venn diagram in Figure 1E illustrates that among the somatic mutations identified in patient numbers 2, 3, 5, and 8, 37%, 78%, 69%, and 77% of these mutations were consistently observed in both the parental tumor and corresponding GBO. Additionally, the correlation of variant allele frequency (VAF), including subclone mutations with low VAF to loss-of-heterozygosity mutations with VAFs close to 1, was significant (Figure 1F, $R = 0.43$, $p < 0.001$), indicating that the GBO accurately recapitulated the somatic mutation landscape of the tumor tissue. Detailed mutation information is provided in Table S4. The patterns of copy number gains and losses occurring in the exon regions of each chromosome were also similarly observed across the GBOs and parental tumor tissues (Figure S1C). Additionally, mutational signatures based on somatic mutations demonstrated comparable patterns between GBM and GBOs (Figure S1D). Furthermore, a strong correlation in mRNA expression between GBOs and their parental tissues confirms that GBOs accurately preserve the transcriptional profiles of the parental tumors (Figure 1G). In summary, important and heterogeneous genomic features of tumor tissues, including somatic mutations, VAF, mutational signatures, and CNVs, were maintained in GBOs along with RNA expression patterns.

GBOs predict personalized TMZ response and progression-free survival of GBM patients

A total of 18 GBOs, including 16 GBOs derived from primary GBM cases and 2 recurrent GBOs (GBO-019 and 061, recurrent samples from GBO-005 and 030, respectively), were evaluated for TMZ sensitivity using the GBO-DST. The half-maximal inhibitory concentration (IC_{50}) values and the area under the curve (AUC) were determined based on drug-response curve data across a range of TMZ concentrations, from 0.0005 to 500 μ M. Notably, GBO-001, 005, 008, 024, and 052 demonstrated less than 50% viability at 50 μ M, whereas other GBO lines did not achieve similar sensitivity (Figure 2A). In addition, the AUC of TMZ-sensitive GBOs was significantly lower than that of TMZ-resistant GBOs (Figure 2B, $p = 0.0002$). Based on the

Figure 1. Establishment and characterization of GBOs from patient-derived glioblastoma tissues

- (A) A schematic diagram outlining the process of GBO generation from glioblastoma patient tissues.
 (B) Morphological comparison of GBOs and parental tumor tissues. Representative H&E-stained images of GBOs (bottom row) and their corresponding parental tumor tissues (top row) exhibit shared morphological features. Scale bar, 50 μ m.
 (C) Immunohistochemical characterization of GBOs and parental tissues. Confocal images showing IHC staining for SOX2 (green), GFAP (white), OLIG2 (red), and Hoechst (blue) in parental and GBO tissues from patients 002, 003, 005, and 008. Scale bar, 50 μ m.
 (D) Genomic landscape of glioblastoma tissues and GBOs. OncoPrint displaying mutation profiles of frequently altered genes (>33% frequency) across four glioblastoma tissues and five GBOs, including one recurrent GBO (upper panel). Mutation frequencies are shown as percentages on the right, and mutation types are color coded. Clinical annotations for each patient sample, including patient number, sample type, extent of tumor resection, and MGMT methylation status (lower panel).
 (E) Venn diagram of somatic mutations. Overlapping somatic mutations between GBOs and parental tissues are shown for patients 002, 003, 005, and 008. The number of shared somatic mutations is indicated.
 (F) Variant allele frequency (VAF) correlation. Scatterplot illustrates the correlation of VAFs between GBOs and parental tissues (overall Spearman correlation coefficient = 0.43, $p < 0.001$). Note that the sample from P-002 was not suitable for VAF correlation due to low purity.
 (G) Transcriptomic correlation between GBOs and parental tissues. Contour plots showing strong correlations in mRNA expression profiles between GBOs and their corresponding tumor tissues derived from five patients.

See also Figure S1 and Tables S1, S2, S3, and S6.

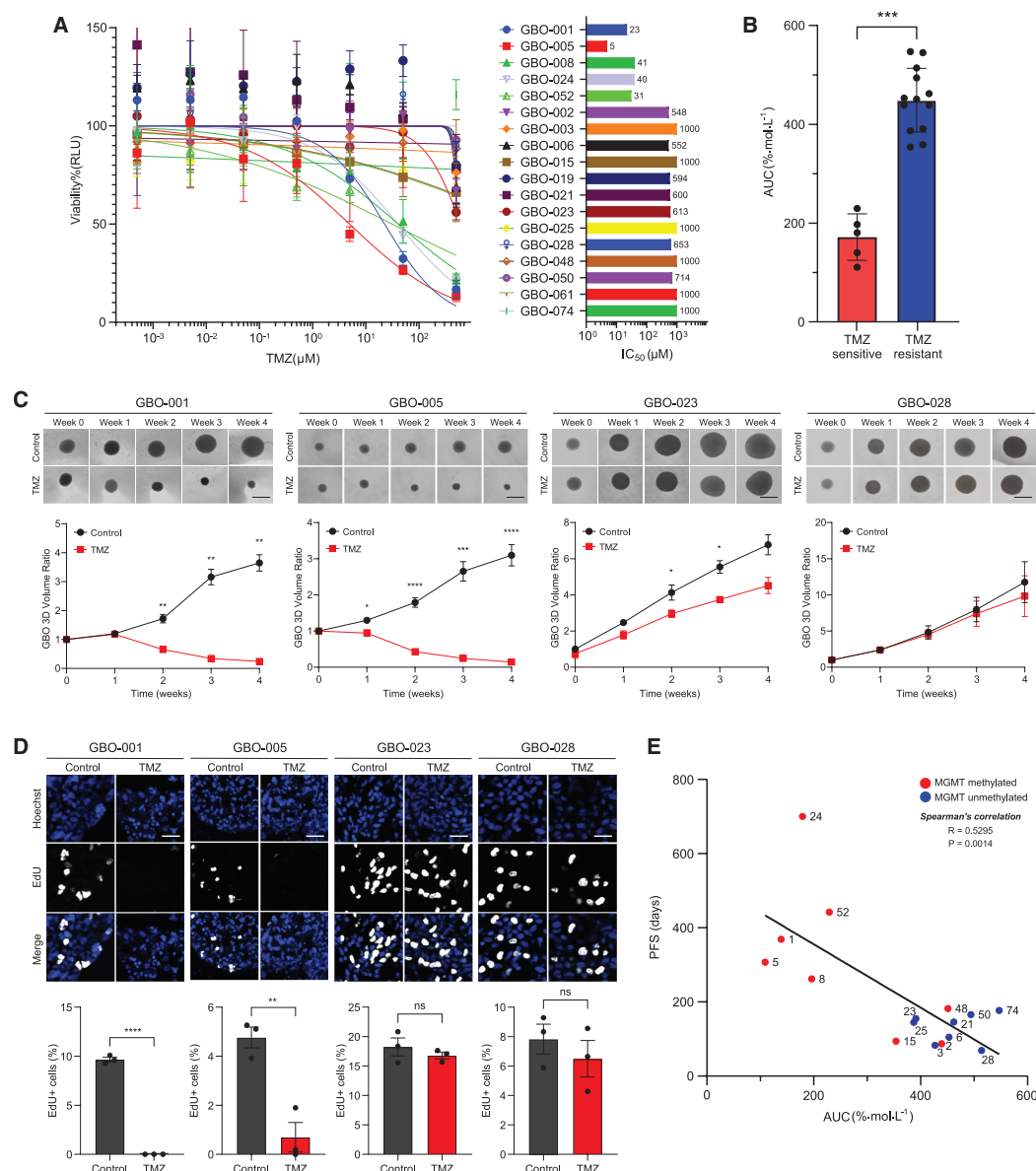


Figure 2. The GBO-DST platform predicts TMZ response of GBM patients

(A) Dose-response curves show cell viability as a function of TMZ concentration for 18 GBOs (left). The histogram (right) displays the IC_{50} values for each GBO. (B) GBOs were categorized into two groups based on area under the curve (AUC) values assessed by GBO-DST ($n = 5$ for TMZ sensitive, $n = 13$ for TMZ resistant; unpaired t tests, $p = 0.0036$). (C) Microscopic images of GBOs and growth curves over time following TMZ ($100 \mu\text{M}$) and DMSO (control) treatments. Scale bars, $500 \mu\text{m}$. Statistically significant time points are marked with an asterisk (*). Values represent mean $\pm$ SEM ($n = 4$ for each condition). Statistical significance was determined using multiple unpaired t tests. p values for significant time points are described in Table S6. (D) Confocal images of EdU staining (white) in GBO sections from (C). Scale bars, $50 \mu\text{m}$. Quantification of EdU+ cells is shown. Values represent mean $\pm$ SEM ($n = 3$ for each condition; p values for each condition are described in Table S6). Statistical significance was determined using unpaired t tests. (E) A significant correlation is observed between PFS times of patients and the IC_{50} values of their corresponding GBOs ($n = 18$ patients; Spearman's correlation, $r = 0.6413$, $p = 0.0006$).

ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. See also Figure S2 and Tables S4 and S6.

GBO-DST, GBO-001, 005, 008, 024, and 052 were designated as candidate TMZ-sensitive GBOs (Figure 2B).

To validate the GBO-DST results, growth curves of GBOs following treatment with 100 μ M TMZ were generated. Consistent with the GBO-DST findings, the TMZ-sensitive GBO line exhibited a significant reduction in size after TMZ treatment. In contrast, the TMZ-resistant GBO line remained unchanged even after 4 weeks of TMZ treatment (Figure 2C). Furthermore, we found that this size alteration was due to GBO proliferation as assessed by EdU assay. EdU, a well-established thymidine analog, is incorporated into newly synthesized DNA, allowing the evaluation of cellular proliferation by measuring the EdU incorporation ratio. In the presence of 100 μ M TMZ for 2 weeks, GBOs were incubated with 10 μ M EdU for 6 h to label proliferating cells. Quantification of EdU⁺ proliferating cells demonstrated a significant decrease in the TMZ-sensitive GBO, but this effect was not observed in the TMZ-resistant GBOs (Figure 2D).

Finally, correlation between GBO-DST results and survival outcomes, including progression-free survival (PFS) and overall survival (OS), were evaluated. All patients in the clinical validation cohort underwent the standard GBM treatment regimen, consisting of TMZ-based CCRT and adjuvant chemotherapy, and reached primary endpoints. The median estimated PFS and OS for the cohort were 150.0 days (95% confidence interval [CI] = 116.7–183.3 days) and 327 days (95% CI = 0–667.6 days), respectively (Figures S2A and S2B). Since TMZ was the only chemotherapeutic agent exposed during the primary treatment period before tumor progression, this cohort is well suited to correlate TMZ sensitivity testing results with clinical outcome, particularly PFS. There was a significant negative correlation between the AUC value of GBOs and the PFS of corresponding patients (Figure 2E, $p = 0.0014$, $Rho = 0.5295$). These findings suggest that GBO-DST can categorize GBOs as TMZ sensitive or TMZ resistant and may serve as a predictive tool for PFS, reflecting TMZ responsiveness in GBM patients.

GBO-DST better predicts personalized response compared to MGMT methylation status

Clinically, well-established factors for predicting patient survival in GBM include age, performance status, extent of resection, and MGMT methylation.^{3,34} Among these, MGMT methylation is the only biomarker currently used in clinical practice to predict responsiveness to TMZ.³⁵ However, compared to MGMT methylation and other clinical factors, TMZ sensitivity determined through GBO-DST proved to be the most reliable predictor of PFS in this cohort (Figure 3A). Notably, all GBO-DST results aligned with the clinical response to TMZ. The median actuarial PFS of the TMZ-sensitive group (369.0 days, 95% CI = 235.9–502.1 days) was significantly longer than that of the TMZ-resistant group ($p = 0.0025$, 146.0 days, 95% CI = 86.7–205.3 days) (Figure 3B).

MGMT methylation status was concordant with TMZ responsiveness from GBO-DST in 14 of 18 cases (77.8%), including the TMZ-sensitive and MGMT methylated group ($n = 5$, GBO-001, 005, 008, 024, and 052) and the TMZ-resistant and MGMT unmethylated group ($n = 9$, GBO-003, 006, 021, 023, 025, 028, 050, 061, and 074). The remaining group, consisting

of 4 cases (GBO-002, 015, 019, and 048), exhibited discordance between MGMT status and GBO-DST, with MGMT methylation but TMZ resistance (Table S4). Notably, no cases demonstrated TMZ sensitivity when the MGMT promoter was unmethylated. The clinical course of representative cases from each group is shown in Figure 3C. Interestingly, all three representative tumors were located in the right temporal lobe, underwent complete surgical resection, and received CCRT. Despite these similarities, the clinical outcomes varied significantly due to differences in TMZ responsiveness. In the cases of GBO-001 and GBO-023, the MGMT status and TMZ responsiveness were concordant. Patient-001 (MGMT methylated and TMZ sensitive) exhibited prolonged PFS, while patient-023 (MGMT unmethylated and TMZ resistant) showed a relatively short PFS, both consistent with expectations. However, in case 15, despite the MGMT promoter being methylated, which typically predicts good responsiveness to TMZ, the tumor recurred as early as the end of CCRT, resulting in a short PFS. Interestingly, GBO-DST classified GBO-015 as TMZ resistant, which aligns with the observed clinical outcome. These findings suggest that GBO-DST may predict TMZ responders more accurately than MGMT status alone.

Transcriptomic analysis reveals potential mechanisms underlying both innate and acquired TMZ resistance in GBOs with MGMT methylation

To further investigate the molecular mechanisms underlying TMZ responsiveness of GBO, we conducted an RNA-seq-based transcriptomic analysis across different GBO lines. RNA-seq data were obtained from seven GBO lines (GBO-001, 002, 003, 005, 006, 008, and 019). The comparison between TMZ-resistant and TMZ-sensitive primary GBOs revealed significant transcriptional differences. Differentially expressed genes (DEGs) with highly significant fold changes were identified, and the top 50 DEGs in TMZ-resistant and TMZ-sensitive GBOs were visualized (Figure 4A). Several genes upregulated in TMZ-resistant GBOs, including *PTPRZ1*, *L1CAM*, *MAPT*, and *GPM6A*, are known to contribute to tumor invasiveness, cell adhesion, and resistance to chemotherapy.^{36–39} Gene set enrichment analysis using the Hallmark gene set was performed to identify the enriched biological pathways associated with TMZ resistance. The enrichment plot revealed a significant upregulation of DNA repair pathways in TMZ-resistant GBOs (Figure 4B).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of genes with fold changes >2 in TMZ-resistant samples revealed significant enrichment in pathways related to cell adhesion, axon guidance, and extracellular matrix interactions (Figure 4C). Similarly, Gene Ontology (GO) enrichment analysis of these DEGs further supported these findings by identifying significant activation of pathways associated with synapse organization, axonogenesis, and cell-cell adhesion processes in the TMZ-resistant GBOs (Figure S3A). These results suggest that TMZ-resistant GBOs are characterized by enhanced cell communication and reinforced structural integrity, potentially contributing to their ability to evade therapeutic interventions.⁴⁰

Furthermore, comprehensive transcriptomic analysis was performed to elucidate potential mechanisms of TMZ resistance in

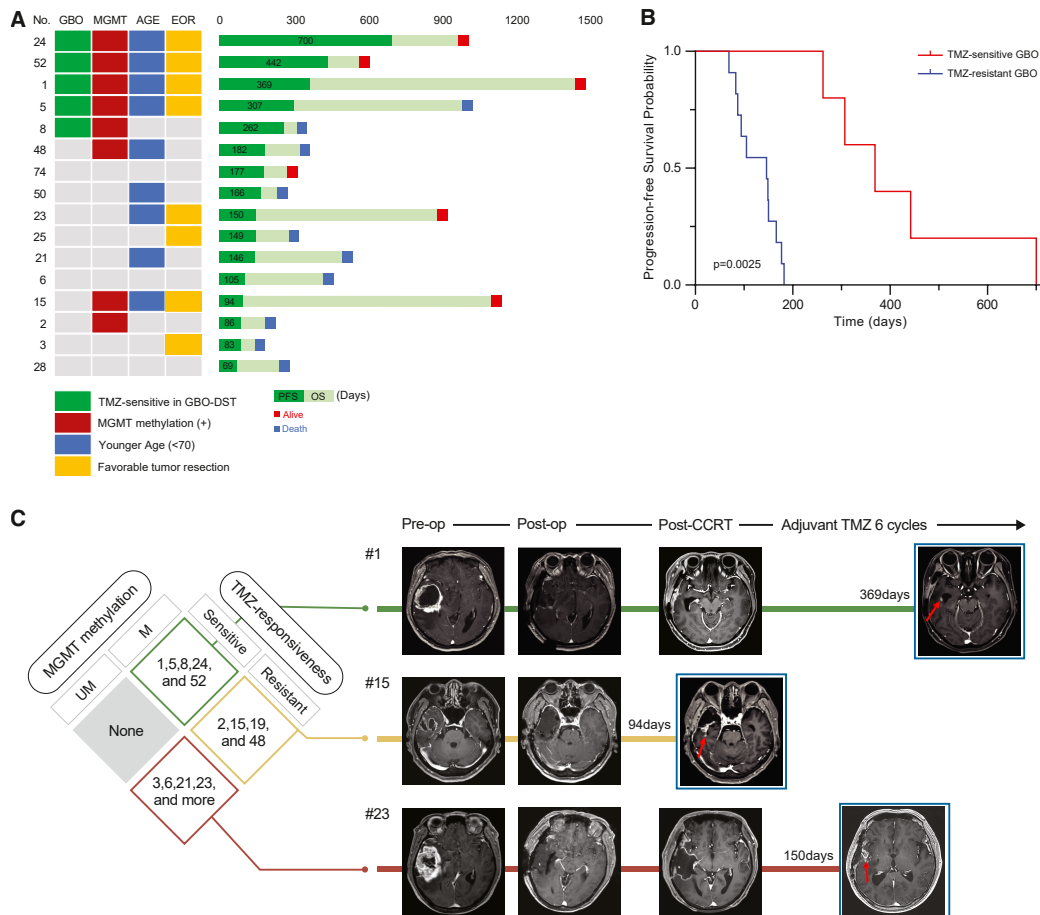


Figure 3. Correlations between TMZ sensitivity predictions using GBO-DST and patient outcomes

(A) Swimmer plot showing individual patient survival outcomes, clinical characteristics, and TMZ responsiveness as determined by GBO-DST. Prognostic factors, including MGMT methylation status, age, and extent of resection, are also described. Notably, TMZ responsiveness of GBOs correlates more strongly with PFS than any other factor examined.

(B) Kaplan-Meier curves demonstrate that patients predicted to be TMZ sensitive based on GBO-DST exhibit significantly prolonged survival compared to the TMZ-resistant group ($n = 5$ for TMZ sensitive, $n = 13$ for TMZ-resistant, log rank test, $p = 0.0025$).

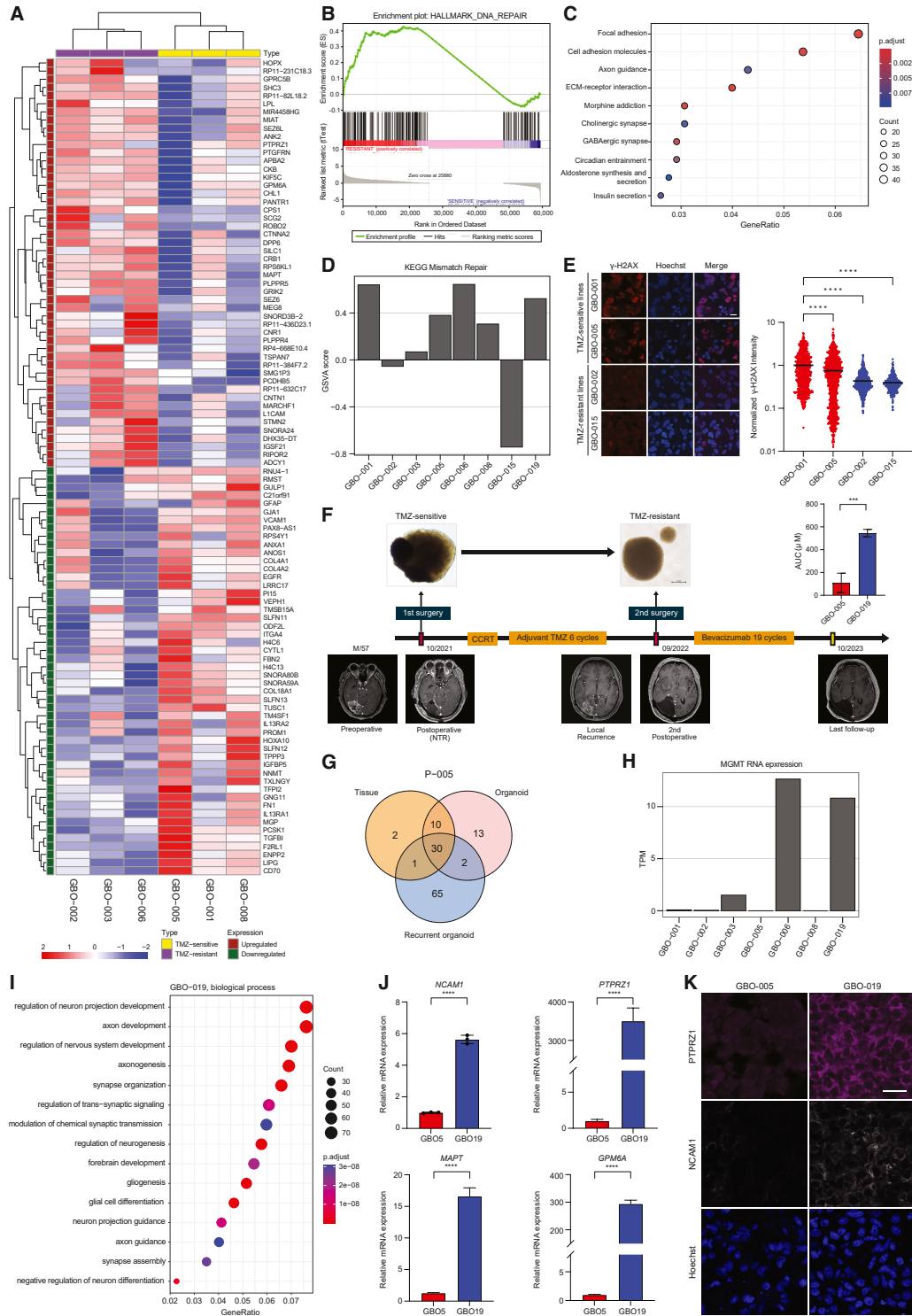
(C) Patients were categorized into three groups based on MGMT methylation status and TMZ responsiveness assessed by GBO-DST. Notably, no patients were classified as MGMT unmethylated while being TMZ sensitive. Representative cases from each group are presented with their clinical courses. In all cases, the tumors were located in similar regions, and favorable resection outcomes were achieved through surgery. However, patient outcomes differed markedly and aligned more closely with GBO-DST results than with MGMT status.

See also Table S6.

GBOs with MGMT methylation. KEGG pathway-based gene set variation analysis revealed that GBO-002 and 015 exhibited reduced expression of the mismatch repair (MMR) system compared to other GBOs (Figure 4D). We also confirmed the downregulation of MMR-related mRNAs by qPCR (Figure S3B). MMR deficiency is known to impair the recognition and processing of mismatched or damaged single-stranded DNA stretches, thereby preventing the generation of DNA double-strand breaks (DSBs) at replication forks.⁴¹ Since DSBs are critical for activating apoptotic pathways in response to TMZ, their absence may contribute to the development of TMZ resistance.⁴² To validate this, we conducted immunostaining against

γ -H2AX, a well-established marker for DSBs, in TMZ-sensitive lines (GBO-001 and GBO-005) and TMZ-resistant lines with reduced expression of the MMR system (GBO-002 and GBO-015). As expected, GBO-001 and GBO-005 displayed widespread DSBs following TMZ treatment, whereas GBO-002 and GBO-015 did not. These findings suggest that the loss of MMR may represent a potential mechanism of intrinsic resistance, as it minimizes replication-associated DNA damage, thereby enabling tumor cells to evade the innate immune response typically triggered by DNA-damaging agents.

In addition, GBO-019, derived from a recurrent tumor sample from the same patient as GBO-005, exhibited TMZ resistance



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despite sharing the same MGMT promoter methylation status as GBO-005 (Figure 4F). WES revealed 65 somatic variants in GBO-019 that were absent in GBO-005 (Figure 4G). These mutations are likely treatment induced, as reflected by their significantly lower VAF in GBO-019 compared to the common mutations shared by both GBOs (Figure S3C). Notably, the mechanism underlying the acquired resistance of GBO-019 to TMZ was also attributed to the elevated expression of MGMT transcripts (Figure 4H). MGMT expression was undetectable in GBO-005, correlating with its sensitivity to TMZ, whereas GBO-019 showed increased MGMT transcript levels despite MGMT promoter methylation. Although methylation-specific PCR confirmed MGMT promoter methylation in both samples in a clinical setting, this discrepancy suggests a potential epigenetic mechanism underlying TMZ resistance in the recurrent tumor. Transcriptomic analysis revealed DEGs between GBO-005 and GBO-019, with GO enrichment highlighting the activation of pathways related to axonogenesis and synapse organization, suggesting enhanced invasiveness in the recurrent sample (Figure 4I). To validate the transcriptomic analysis results, we assessed the expression of genes related to axonogenesis and cell adhesion, such as *NCAM1*, *PTPRZ1*, *MAPT*, and *GPM6A*, using qPCR (Figure 4J). The results showed that these genes were significantly upregulated in GBO-019 compared to GBO-005. Additionally, IHC staining was performed to evaluate the protein expression of cell adhesion markers, including *PTPRZ1* and *NCAM1*, in GBO-005 and GBO-019. Consistent with the qPCR results, *PTPRZ1* and *NCAM1* were more highly expressed in GBO-019 than in GBO-005 (Figure 4K). Based on these observations, we propose that enhanced expression of axonogenesis- and cell-adhesion-related genes together with epigenetic control of MGMT may underlie the increased TMZ resistance observed in GBO-019.

The GBO-DST simulates the result of clinical outcomes and identifies potential drug candidates for GBM treatment

Our findings indicate that the GBO-DST platform is a robust and practical tool for drug testing, with the capability to elucidate underlying mechanisms of drug response. Next, we aimed to evaluate whether GBO-DST could serve as a preclinical model for replicating responses to drugs beyond TMZ.

Through a comprehensive literature review, we selected four FDA-approved drugs recently investigated in clinical trials targeting GBM and/or metastatic brain tumors: regorafenib, bevacizumab, olaparib, and lazertinib. First, regorafenib, a multi-kinase inhibitor, is a targeted therapy agent that demonstrated a survival benefit in a recent phase 2 clinical trial.⁴³ Bevacizumab, a monoclonal antibody against the vascular endothelial growth factor A (VEGF-A), showed efficacy in tumor stabilization and prolongation of PFS but failed to improve OS in large-scale prospective trials.^{44,45} Olaparib, a poly(ADP-ribose) polymerase (PARP) inhibitor, is approved for treating breast, ovarian, and other tumors with *BRCA1* or *BRCA2* mutations. Although it does not penetrate the blood-brain barrier (BBB) and is not used as a monotherapy for GBM, it is being investigated in clinical trials in combination with radiation therapy due to its potential role as a radiosensitizer.^{46–48} Lastly, lazertinib, a third-generation EGFR inhibitor, was selected as a candidate for drug testing due to its ability to penetrate the BBB and its demonstrated efficacy in a recent clinical trial for metastatic brain tumors, offering a distinct advantage over earlier generations of EGFR inhibitors.⁴⁹

Four test drugs, along with the control drug lomustine, were used for drug screening on the established GBOs (Figures 5A and 5B). Lomustine, a DNA-alkylating agent similar to TMZ, showed effective toxicity in TMZ-sensitive GBOs (GBO-001

Figure 4. Transcriptomic analysis highlights molecular pathways related to innate and acquired TMZ resistance in GBOs with MGMT methylation

- (A) Heatmap illustrating gene expression levels across six GBOs (three TMZ sensitive and three TMZ resistant). The top 50 genes identified through hierarchical clustering are labeled on the right.
- (B) GSEA using the hallmark gene set reveals significant enrichment of the DNA repair pathway in TMZ-resistant GBOs.
- (C) GO analysis shows that genes associated with cell adhesion, axon guidance, and extracellular matrix receptor interaction are upregulated in TMZ-resistant GBOs.
- (D) GSVA analysis of the KEGG mismatch repair (MMR) pathway indicates that the MMR pathway is downregulated in GBO-002 and GBO-015, contributing to TMZ resistance.
- (E) Immunohistochemical characterization of TMZ-sensitive and TMZ-resistant GBOs. Confocal images of γ -H2AX (red) and Hoechst (blue) staining in GBO-001 and 005 (TMZ-sensitive lines) and GBO-002 and 015 (TMZ-resistant lines). Scale bar, 10 μ m. The scatterplot shows γ -H2AX intensity based on all cells within each organoid, normalized to GBO-001. The bars represent mean values. (GBO-002 $p < 0.0001$, GBO-005 $p < 0.0001$, GBO-015 $p < 0.0001$). Statistical significance was determined using ordinary one-way ANOVA.
- (F) Clinical course of patient P-005: GBO-005, derived from the first surgery, was identified as TMZ sensitive through GBO-DST. After completing the standard treatment regimen, the tumor locally recurred. GBO-019, established from the second surgery, was found to be TMZ resistant based on AUC values from the dose-response curve, in contrast to the original GBO-005. Statistical significance was determined using z test ($p < 0.001$).
- (G) Venn diagram showing the number of overlapping somatic variants between tumor tissue, organoid, and recurrent organoid of patient P-005.
- (H) MGMT mRNA expression levels correspond to MGMT methylation status in all GBOs except GBO-019. Notably, GBO-019 shows high MGMT RNA expression despite having a methylated MGMT promoter.
- (I) GO analysis comparing GBO-005 and GBO-019 reveals upregulation of pathways related to axon and synapse development and organization in GBO-019, which are associated with tumor invasiveness.
- (J) Validation of gene expression in GBO-005 and GBO-019 by qPCR. Values are normalized to β -actin expression and presented as mean $\pm$ SD ($n = 3$ technical replicates; *NCAM1* $p < 0.0001$, *PTPRZ1* $p < 0.0001$, *MAPT* $p < 0.0001$, *GPM6A* $p < 0.0001$). Statistical significance was determined using unpaired t tests.
- (K) Representative confocal images of *PTPRZ1* and *NCAM1* expression in GBO-005 and GBO-019. Scale bar, 100 μ m.

**** $p < 0.0001$, See also Figure S3 and Table S6.

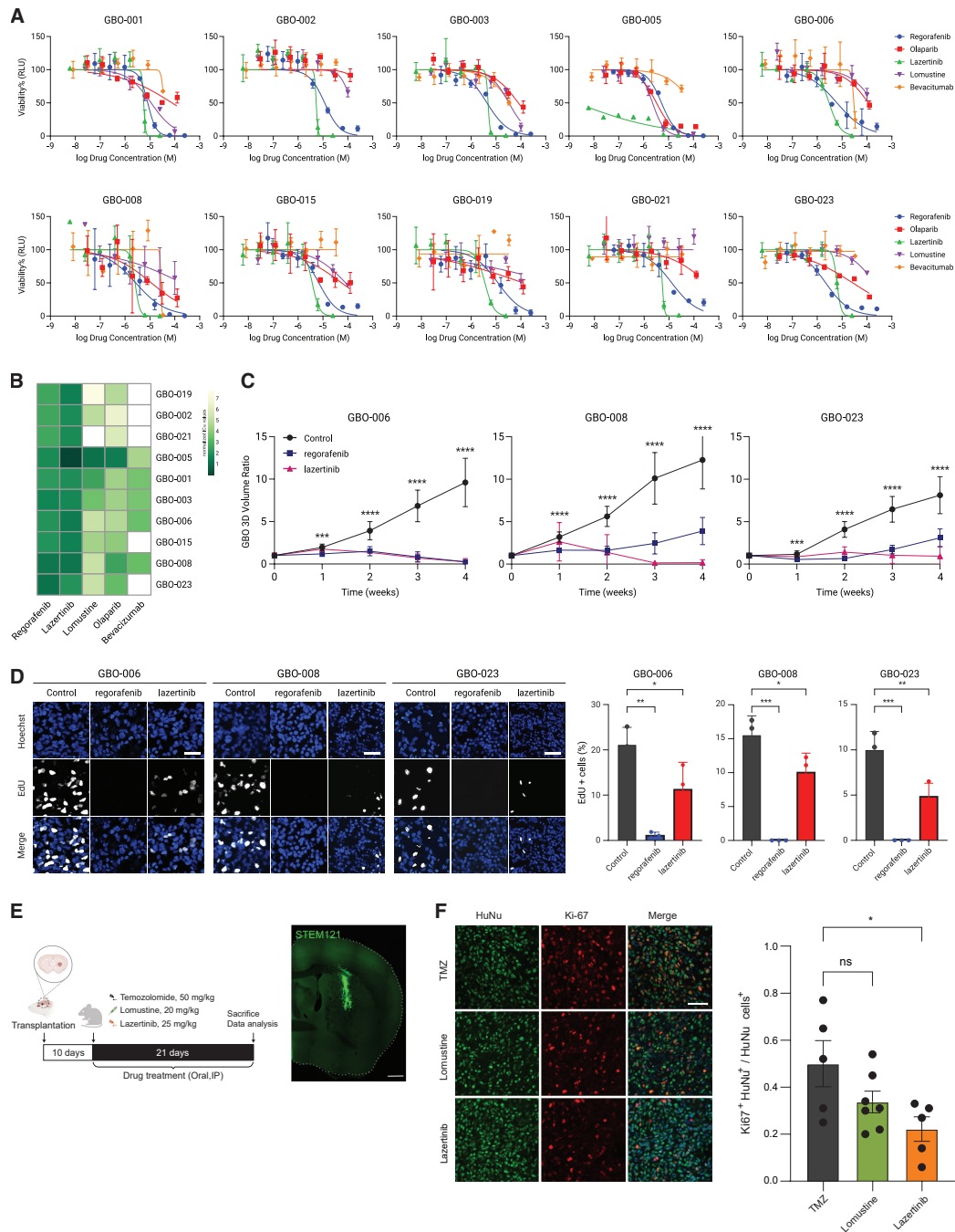


Figure 5. GBO-DST can serve as a predictive platform for clinical responses in drug repurposing

(A) Dose-response curves showing cell viability for eight GBOs treated with five drugs: regorafenib, olaparib, lazertinib, lomustine, and bevacizumab. (B) Heatmap displaying IC_{50} values for 10 GBOs treated with the five drugs mentioned in (A). The normalized IC_{50} values are calculated as $\ln(IC_{50} [\mu M] + 1)$. White cells indicate “not applicable” (N/A). (C) Growth curves over time for GBOs treated with regorafenib (10 μM), lazertinib (10 μM), and DMSO (control). Statistically significant time points are marked with an asterisk (*). Values represent mean $\pm$ SD ($n = 12$ for GBO-006 and GBO-023, $n = 16$ for GBO-008). Statistical significance was determined using multiple unpaired t tests. p values for significant time points are described in Table S6.

(legend continued on next page)

and 005), suggesting that the GBO-DST is effective for evaluating alkylating agents beyond TMZ. Olaparib only showed selective efficacy against GBO-005, and bevacizumab was ineffective against all GBO lines. Consistent with the findings from clinical trials, olaparib is presumed to be ineffective as a monotherapy.^{46–48} Furthermore, although bevacizumab exhibits selective activity in clinical settings, it is not suitable for evaluating its drug efficacy in the GBO-DST because VEGF receptors are not expressed in GBOs, unlike in the original GBM (Figure S4).

Notably, regorafenib and lazertinib were universally effective across all GBO lines. Regorafenib and lazertinib show lower IC₅₀ values compared to other drugs evaluated in this study (Figure 5B). For further investigation, regorafenib and lazertinib were tested on three GBO lines (GBO-006, GBO-008, and GBO-023). Treatment of these GBOs with 10 μ M regorafenib and lazertinib for 4 weeks resulted in a significant reduction in GBO size (Figure 5C). The drugs were effective regardless of their previously identified responses to TMZ. Additionally, after 2 weeks of drug treatment, a significant reduction in EdU⁺ proliferating cells was observed in the treated groups compared to the controls (Figure 5D). Because regorafenib has been approved for GBM treatment, its efficacy as determined by GBO-DST supports the ability of this platform to predict personalized drug responses.⁴³ Notably, although lazertinib is not currently used to treat GBM, this finding demonstrates the capacity of GBO-DST to identify therapeutic candidates. Collectively, these results suggest that GBO-DST can replicate clinical responses to selective agents and may serve as a platform for drug screening and the discovery of potential therapeutics.

To evaluate the *in vivo* efficacy of potential drug candidates, we transplanted GBO-023—previously characterized as TMZ resistant but sensitive to lazertinib—into the brains of immunodeficient mice. For transplantation experiments, GBOs were mechanically dissociated and injected into the mouse brain. After a 10-day stabilization period, mice were treated with TMZ, lomustine, or lazertinib for 21 days. Subsequently, the mice were sacrificed, and the proliferation of transplanted GBOs was assessed (Figure 5E). HuNu was used as a marker of human cell origin to distinguish GBO cells from the host mouse brain, while Ki-67 served as a proliferation marker to evaluate tumor growth. The proliferation index was calculated as the ratio of Ki-67⁺HuNu⁺ cells to the total number of HuNu⁺ cells. Comparative analysis revealed that lazertinib exerted the strongest inhibitory effect among the treatment groups (Figure 5F). These findings demonstrate that *in vivo* lazertinib treatment effectively suppresses the proliferation of TMZ-resistant GBOs, highlighting its potential as an alternative therapeutic option.

DISCUSSION

Since the introduction of TMZ as the standard treatment regimen for GBM, efforts to identify effective drugs have largely been unsuccessful.^{2,3} Unfortunately, over 50% of GBM patients fail to respond to TMZ therapy, and there are limited biomarkers beyond MGMT status to predict the response to TMZ.^{10,50} The primary challenges in GBM treatment stem from the tumor's inherent characteristics, such as its heterogeneity and rapid evolution during treatment. Additionally, the relative rarity of GBM, coupled with its distinct microenvironment, poses significant challenges for drug development and the execution of clinical trials.⁷ These factors underscore the critical need to develop and refine preclinical models using PDOs in GBM research.^{31,51} Several authors have successfully reported the value of PDO-based DST to predict clinical responses.^{51–54} For example, Wang et al. demonstrated that DST using lung cancer organoids accurately predicted clinical responses to treatment in 83.3% of patients.²² Similarly, Yao et al. confirmed that PDOs from locally advanced rectal cancer patients recapitulated clinical responses with a high accuracy of 84.4%.²³ Deligne et al. tested PDOs against standard-of-care and other therapies, identifying ONC201 as a selective drug for pediatric glial tumors.⁵² Our study highlights that DST using GBO is a highly effective approach, demonstrating strong correlations with clinical outcomes and enabling efficient drug screening and candidate identification. We further validated these candidates using an *in vivo* GBO transplantation model, offering alternative therapeutic options to advance personalized medicine for GBM.

To paraphrase the famous opening line of *Anna Karenina*, the results of GBO-DST can be described as follows: “All sensitive GBOs are alike; each resistant GBO resists in its own way.” In other words, all sensitive GBOs had MGMT methylation, while resistant GBOs had their own drug-resistant mechanisms including MGMT-independent pathways, MMR deficiency, enhanced cell-cell communication, or epigenetic alterations. In this study, all patients' clinical responses matched the result of GBO-DST in terms of TMZ sensitivity, while only 75% of GBOs exhibited responses aligned with their MGMT methylation status. This discrepancy can likely be attributed to the cell composition of organoids, which may be influenced by intratumoral heterogeneity and regional sampling bias in the parental tumor.⁵¹ Notably, three GBOs demonstrated TMZ resistance in both the GBO-DST and real-world clinical settings, despite exhibiting MGMT methylation. Transcriptomic analyses further revealed that TMZ-resistant GBOs harbored diverse drug resistance mechanisms. However, assessing TMZ sensitivity through GBOs bypasses the need to investigate the diverse mechanisms

(D) Confocal images of EdU staining (white) in GBO sections from (C). Scale bars, 50 μ m. Quantification of EdU⁺ cells is shown. Values represent mean $\pm$ SD ($n = 3$ for each condition; p values for each condition are described in Table S6). Statistical significance was determined using ordinary one-way ANOVA.

(E) Schematic illustration of the transplantation experiment. Representative confocal images of STEM121 staining (green) in mouse brain sections demonstrate successful GBO transplantation. Scale bar, 100 μ m.

(F) Confocal images of Ki-67 (red) and HuNu (green) staining in transplanted mouse brain sections. Scale bar, 50 μ m. Quantification of proliferating cells is shown as the ratio of Ki-67⁺HuNu⁺ cells to total HuNu⁺ cells. Values represent mean $\pm$ SEM ($n = 5$ mice per group; lomustine, $p = 0.1333$; lazertinib, $p = 0.0308$ compared with TMZ). Statistical significance was determined by one-way ANOVA.

ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. See also Figure S4 and Table S6.

underlying individual resistance. A recent study conducting a comprehensive proteogenomic analysis of 123 matched longitudinal GBM samples suggested that epigenetic mechanisms, including protein inactivation, play a critical role in TMZ responsiveness and tumor recurrence, alongside genetic mutations.⁵⁵ Therefore, the findings of GBO-DST appear to provide a more reliable and efficient method for predicting TMZ responsiveness in GBM patients, offering significant advantages over genetic approaches such as relying solely on MGMT methylation status.

For the PDOs to serve as a robust preclinical trial platform, they must exhibit sufficient diversity to represent a wide range of patients, akin to clinical trials. Therefore, the success rate of PDO formation is a critical factor. While the success rate in other tumor types varies widely, our study achieved a rate of 61.0%.^{22,56,57} Interestingly, imaging findings suggest that aggressive or extensive GBM may negatively impact GBO formation. Although a detailed mechanistic analysis of this phenomenon is beyond the scope of this study, it may have significant implications for optimizing GBO platforms and refining culture conditions in the future. In addition, while GBM is a relatively rare cancer, a major limitation of this study is the small size of the clinical validation cohort. Moreover, the limited number of cases precluded statistical determination of a reference IC₅₀ and AUC value for assessing TMZ sensitivity, which represents another limitation of the study. In this study, TMZ sensitivity was defined as < 50% viability at 50 μM, which is lower than the IC₅₀ values (100–400 μM) typically reported in GBM cell line-based studies.⁵⁸ As our study employed patient-derived GBOs, which exhibit different proliferation kinetics from established cell lines, we adopted our previously established organoid-based DST protocol developed in lung cancer.²⁴ Under these conditions, prolonged TMZ exposure (144 h) resulted in IC₅₀ values below 50 μM in all sensitive GBOs, whereas resistant GBOs consistently exceeded 500 μM, supporting the use of a 50 μM cutoff for specific identification of sensitivity. Furthermore, our approach is consistent with other recent studies that applied TMZ concentration in a similar range.^{51,53,59} Nonetheless, additional validation in larger independent cohorts will be necessary to determine whether this cutoff optimally predicts clinical responses. In addition, establishing a larger cohort is essential to ensure genetic diversity within the platform and to facilitate robust clinical validation. To address this, we are actively enrolling patients and biobanking samples from multiple tertiary brain tumor centers (NCT06782984). Another critical issue in the clinical application of the GBO-DST platform is the time required from surgical resection to obtaining drug test results. In this study, the establishment of GBOs and their growth to a sufficient size for both drug testing and biobanking generally required 4 weeks. Since GBM patients typically initiate CCRT around 4 weeks following surgery, identifying drug candidates within this time frame could enable the efficient clinical implementation of the personalized GBO-DST platform. Nevertheless, it remains crucial to optimize culture conditions to further shorten the required time, and to develop approaches that enable drug testing with smaller amounts of GBOs, both of which are essential for clinical applicability.

The therapeutic potential of regorafenib in GBM has been previously explored, most notably in the REGOMA phase 2 trial.⁴³

Our findings build upon this established knowledge by demonstrating the feasibility of using GBO to assess regorafenib sensitivity at an individual level, thereby supporting a possible role of GBO-DST in personalized medicine. On the other hand, the promising performance of lazertinib in the GBO-DST and subsequent validation in an *in vivo* GBO orthotopic transplantation model provides a strong rationale for its potential applicability in real-world settings as an alternative therapeutic option. Following the failure of phase 2 clinical trials with gefitinib, no clinical trials have yet investigated third-generation EGFR TKIs for GBM.^{60,61} However, CNS-penetrant EGFR TKIs suggest their promise as potential candidates for GBM treatment. Looking ahead, future clinical trials will be essential to fully establish the therapeutic benefit of lazertinib in TMZ-resistant GBM patients. If therapeutics identified by the GBO-DST platform demonstrate clinical efficacy, this strategy could substantially accelerate their translation to patients by reducing reliance on preclinical animal testing and conventional clinical trials. In conclusion, our study demonstrates that GBO-DST as a robust and clinically relevant platform that correlates strongly with patient outcomes, enabling efficient drug sensitivity testing and advancing personalized treatment strategies for GBM. By offering critical insights into the feasibility of identifying promising drug candidates, the GBO-DST demonstrates its utility in expediting the discovery of effective therapies and addressing the urgent need for improved treatment options in GBM.

Limitations of the study

This study has several limitations that should be considered when interpreting the findings. The relatively small cohort size limits statistical power and precludes robust cutoff determination for IC₅₀ or AUC, as well as comprehensive risk factor analyses. This reflects challenges inherent to GBM research, including limited sample availability and modest organoid establishment rates. In addition, the predominance of TMZ-resistant cases and the longer survival of TMZ-sensitive patients reduced the number of evaluable TMZ-sensitive GBOs. The current GBO-DST platform also does not incorporate radiotherapy and, therefore, does not recapitulate the effects of concurrent chemoradiation. Further validation of GBO-DST in larger patient cohorts through prospective studies will be necessary. Finally, the GBO model does not fully capture the TME. The absence of vascular and immune components limits the evaluation of therapies targeting these compartments, and interactions with surrounding neural tissue are not adequately represented. Further development of organoid systems incorporating diverse microenvironmental components will be important to improve the physiological relevance of this platform.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ki-Jun Yoon (kijunyon@kaist.ac.kr).

Materials availability

This study did not generate new materials or unique reagents.

Data and code availability

- The raw whole-exome sequencing and RNA sequencing data generated from patient's sample and patient-derived glioblastoma organoids in this study are deposited in the European Genome-Phenome Archive (EGA) under study ID EGAS50000001221 and dataset ID EGAD50000001741.
- The R script used for the NGS data analysis in this study is available on GitHub (<https://github.com/SGMedical/GBO>).
- Any additional information required to reanalyze the data reported in this work paper is available from the [lead contact](#) upon request.

ETHICS DECLARATIONS

The collection of clinical data and tumor specimens for GBO culture, genetic characterization, drug testing, and further evaluation was conducted following the approval of the Institutional Review Board (IRB) of the Research Ethics Committee at Chungnam National University Hospital, Daejeon, South Korea (IRB no. 2021-08-020-014). Written informed consent was obtained from all patients prior to surgery.

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AUTHOR CONTRIBUTIONS

K.H.K. and K.-J.Y. designed and funded the study. J.P. and M.Q. generated GBOs, and J.P., M.Q., J.-w.O., H.M., and H.Y. performed drug sensitivity tests. E.J.C. and Y.K. conducted sequencing experiments and bioinformatics analysis. S.S., J.P., H.P., and A.L. performed *in vivo* transplantation experiments. J.-H.M. contributed in part to the generation of GBOs. J.P., M.Q., E.J.C., J.-H.M., M.K., H.P., Y.K., H.M., S.-H.K., Y.S., S.M.K., K.S.S., H.-J.O., K.R.C., and K.Y. performed additional experiments and analyzed data. N.-S.K., S.J.J., K.H.K., and K.-J.Y. supervised the research. J.P., M.Q., E.J.C., N.-S.K., K.H.K., and K.-J.Y. wrote the manuscript.

DECLARATION OF INTERESTS

All authors have approved the final manuscript and agreed to its submission.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-SOX2	Santa Cruz	sc-365964; RRID:AB_10843364
Chicken anti-GFAP	Abcam	ab4674; RRID:AB_304558
Rabbit anti-OLIG2	Abcam	ab109186; RRID:AB_10861310
Mouse anti-NCAM1	Abcam	ab9018; RRID:AB_306945
Rabbit anti-PTPRZ1	Abcam	ab126497; RRID:AB_11127912
Anti-Human Nuclear Antigen antibody [235-1]	Abcam	ab191181; RRID:AB_2885016
Mouse Anti-STEM121	Takara	Y40410; RRID:AB_2801314
Rabbit anti-Ki67	Proteintech	27309-1-AP; RRID:AB_2756525
Mouse anti phosphor histone H2A.X (Ser139), clone JBW301	Sigma	05-636; RRID:AB_309864
Alexa 488 donkey-anti-mouse IgG	Jackson ImmunoResearch	715-545-150; RRID:AB_2340846
Alexa 647 donkey-anti mouse IgG	Jackson ImmunoResearch	715-605-150; RRID:AB_2340862
Alexa 594 donkey-anti chicken IgG	Jackson ImmunoResearch	703-585-155; RRID:AB_2340377
Alexa 594 donkey-anti rabbit IgG	Jackson ImmunoResearch	711-585-152; RRID:AB_2340621
Alexa 647 donkey-anti rabbit IgG	Jackson ImmunoResearch	711-605-152; RRID:AB_2492288
Biological samples		
Human glioblastoma tissue	This study	Table S1
Chemicals, peptides, and recombinant proteins		
Hoechst 33342	Invitrogen	Cat #H3570
temozolomide	Sigma	Cat #T2577
temozolomide	Selleckchem	Cat #S1237
Regorafenib	Selleckchem	Cat #S1178
Lazertinib	Selleckchem	Cat #S8724
Olaparib	Selleckchem	Cat #S1060
bevacizumab	Selleckchem	Cat #A2006
Lomustine	Selleckchem	Cat #S1840
TRizol	Invitrogen	Cat #15596018
Critical commercial assays		
DNeasy® Blood & Tissue kit	Qiagen	Cat #69504
SureSelect V6-Post DNA library kit	Agilent Technologies	Cat #G9611C, #5190-8864
TruSeq Standard Total RNA Library Prep Gold Kit	Illumina	Cat # 20020599
NovaSeq 6000 S4 Reagent Kit v1.5 (300 cycles)	Illumina	Cat #20028312
Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647	Thermo Fisher Scientific	Cat #C10640
CellTiter-Glo assay	Promega	Cat #G7570
PrimeScript™ RT Master Mix (Perfect Real Time)	Takara	Cat # RR036A
Deposited data		
RNA-seq data	This paper	Study ID EGAS50000001221 and dataset ID EGAD50000001741
Whole exome-seq data	This paper	Study ID EGAS50000001221 and dataset ID EGAD50000001741

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Glioblastoma organoid lines	This paper	Table S1
Experimental models: Organisms/strains		
NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Wjl} /SzJ	Jackson Laboratory	Strain #:005557 RRID:IMSR_JAX:005557
Oligonucleotides		
Primers, see Table S5	This paper	N/A
Software and algorithms		
R software	R project for statistical computing	https://www.r-project.org/
Prism 8, v8	GraphPad software	https://www.graphpad.com/
FASTP (v0.23.2)	Chen et al. ⁶²	https://www.bioinformatics.babraham.ac.uk/projects/fastqc
Burrows-Wheeler Aligner (v0.7.17-r1188)	Li et al. ⁶³	N/A
Samtools(v1.16.1)	Li et al. ⁶⁴	N/A
Genome Analysis Toolkit (GATK4 v4.2.6.1)	Li et al. ⁶⁴	https://gatk.broadinstitute.org/hc/en-us
ApplyBQSR	Pedersen et al. ⁶⁵	N/A
Ensemble Variant Effect Predictor (v106.1)	McLaren et al. ⁶⁶	N/A
Vcf2maf(v1.6.21)	McLaren et al. ⁶⁶	N/A
CNVkit(v0.9.9)	Talevich et al. ⁶⁷	N/A
deconstructSigs (v1.9.0)	Rosenthal et al. ⁶⁸	N/A
STAR (v2.7.9a)	Dobin et al. ⁶⁹	N/A
RSEM(v1.3.1)	Li et al. ⁷⁰	N/A
Gene Set Variation Analysis (GSVA)	Subramanial et al. ⁷¹	N/A
Gene Set Enrichment Analysis (GSEA)	Hänzelmann et al. ⁷²	N/A
BioRender	–	https://www.biorender.com/
The R script used for the NGS data analysis	This paper	https://github.com/SGMedical/GBO

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Patient sample and data collection

Since August 2021, all patients diagnosed with GBM based on MRI findings have been prospectively enrolled in the local database, with efforts made to establish GBOs. Most patients underwent tumor resection via conventional craniotomy. For cases where the tumor was unresectable, or the patient had poor performance status, brain biopsy was performed using a navigation system (StealthStation S8 system and SureTrak 2 universal tracker instrument, Medtronic). Fresh tumor tissues were placed in a nutrient medium (Hibernate A, TransnetYX) and promptly transported to the laboratory, typically within 12 h.

Tumor samples were initially diagnosed through histologic examination based on the 2021 WHO classification of tumors of the central nervous system (WHO-CNS5), and an integrative diagnosis of GBM was confirmed using next-generation sequencing (NGS) panel sequencing analysis.⁹ Given the inherent difficulty in distinguishing GBM based solely on preoperative imaging from other pathologies, such as metastatic brain tumors or other gliomas, all tumors suspected to be GBM were prospectively included in the GBO generation process. If the histologic diagnosis later revealed that the tumor was not GBM, those GBOs were excluded from further data collection and analysis. Notably, no organoids were successfully cultured from non-GBM tumors using our GBO protocol. The GBO numbers corresponding to these excluded cases were 10 (metastatic brain tumor), 11 (pleomorphic xanthoastrocytoma), 14 (metastatic brain tumor), 17 (recurrent oligodendroglioma), 20 (diffuse astrocytoma, IDH-mutant, grade 4), 38 (diffuse astrocytoma, IDH-mutant, grade 3), 40 (brain metastasis), 54 (diffuse large B-cell lymphoma), 57 (ependymoma), 59 (metastatic melanoma), 63 (diffuse large B-cell lymphoma), and 67 (diffuse astrocytoma, IDH-mutant, grade 3). Sample 62 was derived from the identical patient as sample 61, who presented with multiple lesions involving the right and left hemisphere. Samples 71 and 72 were derived from multiple lesions (cortex, core and ventricle-invading) of the identical patient of 70. These exclusions resulted in gaps in GBO numbering (Table S1).

Clinical data were prospectively collected, including patient demographics, tumor characteristics, surgical outcomes, treatment history, and survival outcomes (Tables S1 and S2). Preoperative MRI images were reviewed for tumor size, location, and infiltrative features such as leptomeningeal disease, multifocal enhancement, and gliomatosis cerebri. Key clinical and tumor characteristics, including initial MRI-based tumor infiltration, were analyzed to identify factors contributing to the failures of GBO formation (Table S3).

The EOR was assessed according to the report from the response assessment in neuro-oncology (RANO) resection group and dichotomized into favorable (supramaximal and maximal contrast-enhancing resection) and unfavorable (submaximal contrast-enhancing resection and biopsy) resection.³⁴ The methylation status of the MGMT promoter was retrieved from pathological reports based on the result of methylation-specific polymerase chain reaction (MS-PCR, Seoul Clinical Laboratories). All patients, except those with poor performance status, underwent a standard temozolomide-based treatment regimen after surgery, including CCRT followed by adjuvant chemotherapy.² The primary clinical endpoints were tumor progression and death. The tumor progression was evaluated following the recently updated criteria from RANO 2.0.⁷³ Statistical analyses were conducted using R software (R Project for Statistical Computing) and Prism (version 8.0, GraphPad Software). Survival analysis and group comparisons were performed using the Kaplan-Meier method with log rank tests. Multivariate analyses were conducted using the Cox proportional hazards model. Spearman's correlation was used to compare nonparametric continuous variables.

GBO generation and biobanking

GBOs were generated as previously described.³³ Following the prompt transportation of the tumor specimen to the laboratory, GBM samples were washed with cold phosphate-buffered saline (PBS) and minced into tumor fragments approximately 500 μ m in diameter using sterilized scalpel blade. A small, randomly selected portion of these fragments was fixed in 4% paraformaldehyde (PFA) for histopathological analysis, while another portion was snap-frozen and stored at -80°C for subsequent DNA and RNA extraction. The remaining fragments were utilized for the generation of GBOs. The complete human GBO culture medium composition was as follows: DMEM-F12 and Neurobasal (1:1 ratio) supplemented with $1\times$ B27 (minus vitamin A), $1\times$ N2, $1\times$ Glutamax (GIBCO, Thermo Fisher Scientific), $1\times$ NEAA (GIBCO, Thermo Fisher Scientific), $1\times$ Penicillin-Streptomycin (GIBCO, Thermo Fisher Scientific), $1\times$ β -Mercaptoethanol (GIBCO, Thermo Fisher Scientific), and 1 mM human recombinant insulin (Sigma-Aldrich). Tumor fragments were transferred to a 15 mL conical tube, washed with cold PBS. The tumor pieces were then cultured on a CO_2 -resistant orbital shaker at 120 rpm in a sterile incubator set to 37°C , 5% CO_2 , and 90% relative humidity, using 4 mL of medium in a 6-well flat-bottom low-attachment plate for a continuous duration of 2–4 weeks. For biobanking, GBOs were dissected into $\sim 300\ \mu\text{m}$ fragments, incubated in a medium containing 10% DMSO for 15 min at room temperature, and transferred to cryovials for long-term storage in liquid nitrogen. After thawing and recovering, GBOs reaching 1 mm in diameter were passaged by dissection into approximately 0.5 mm diameter fragments.

METHOD DETAILS

Animals

Mice were housed in groups of up to five per cage under a 12 h light/12 h dark cycle with free access to food and water. Male and female NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice (NSG mice, 7–10 weeks old; The Jackson Laboratory, RRID: IMSR_JAX:005557) were used for all experiments and randomly assigned to treatment groups. Animals were monitored at least twice per week for body weight and were routinely examined for physical and neurological abnormalities. Mice were housed in standard conditions on a 12-h light/dark cycle in a temperature- and humidity-controlled barrier facility and allowed *ad libitum* access to standard chow and water. All animal experiments were approved by and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Korea Advanced Institute of Science and Technology.

Histology and immunohistochemistry

GBM tissues and GBOs were fixed in 4% PFA for 1 h at room temperature, washed with PBS, and immersed in 30% sucrose overnight at 4°C . Once the tissues or GBOs had sunk, they were transferred to the optimal cutting temperature (OCT, Sakura Finetek) compound for mold embedding and rapidly frozen using dry ice. Sections of 10 μm thickness were obtained using a cryostat and mounted onto glass slides for further processing. For EdU labeling, GBOs were incubated with EdU (10 μM) for 6 h before fixation.

Immunohistochemical (IHC) staining was performed for glial fibrillary acidic protein (GFAP), SRY-box transcription factor 2 (SOX2), oligodendrocyte transcription factor 2 (OLIG2), protein tyrosine phosphatase receptor type Z1 (PTPRZ1), neural cell adhesion molecule 1 (NCAM1), Antigen Kiel 67 (Ki-67) and Phospho Histone H2A.X(Ser139) (γ -H2AX on all tumor tissues and organoids). The sections were treated with a blocking buffer containing 5% donkey serum, 3% bovine serum albumin (BSA), and 0.1% Triton X-100 for 1 h at room temperature. They were subsequently incubated with a primary antibody diluted in blocking buffer (1:400) at 4°C overnight. Following this incubation, the slides were washed three times with PBS, with each washing lasting 10 min, and then incubated with a secondary antibody diluted in blocking buffer (1:1000) at room temperature for 1 h. After three additional washes with PBS (10 min each), the nuclei were stained with Hoechst 33258 (1 $\mu\text{g}/\text{mL}$, Sigma-Aldrich) at room temperature for 5 min. The slides were washed three times with PBS and then mounted with a mounting solution. For EdU detection, Click-iT EdU Alexa Fluor 647 imaging kit (Thermo Fisher Scientific) was used according to the manufacturer's protocol.

For IHC analysis of GBO orthotopic transplantation, perfused brains were fixed in 4% PFA overnight and cryoprotected in 30% sucrose in PBS. Serial coronal sections were prepared using a sliding microtome (SM2010R, Leica Microsystems) and stored at -20°C in antifreeze solution (300 g sucrose, 300 mL ethylene glycol, 500 mL 0.1 M PBS). Prior to immunostaining, sections were washed twice in PBS to remove the antifreeze solution. Sections were incubated at 4°C for 24–48 h with primary antibodies diluted in blocking solution (5% goat serum, 3% BSA, 0.05% Triton X-100 in PBS). After three washes in 0.05% Triton X-100 in PBS, sections

were incubated with secondary antibodies (Thermo Fisher Scientific) at 1:1000 dilution for 2 h at room temperature. Sections were then mounted on Superfrost Plus slides (Thermo Fisher Scientific) and coverslipped with fluorescence mounting medium (Abcam, ab104139). Following staining, images of the slides were captured using a confocal microscope (Nikon AX R Confocal Microscope System, Nikon).

Next-generation sequencing library preparation and sequencing

DNA was extracted from GBM tissues, matched blood samples, and GBOs using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol. Exome sequencing libraries were prepared from DNA samples using the SureSelect V6-Post DNA library Kit (Agilent Technologies). Blood libraries were sequenced to a depth of 100X, while tumor samples were sequenced to a depth of 300X, ensuring sufficient coverage for reliable variant identification. RNA sequencing (RNA-seq) libraries were prepared using the TruSeq Stranded Total RNA Library Prep Gold Kit (Illumina), with an expected output of 20 Gb per sample. Next-generation sequencing was performed on the NovaSeq 6000 platform (Illumina) using paired-end sequencing (2 × 100 bp) with the NovaSeq 6000 S4 Reagent Kit v1.5 (300 cycles).

Genomic data analysis

WES and RNA-seq were both processed using a pipeline built under the Nextflow workflow engine.⁷⁴ The initial quality control and adapter trimming of the raw WES data were performed using FASTP (v0.23.2), followed by FASTQC (v0.11.9) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).⁶² Sequenced reads were aligned to the human reference genome (NCBI build 38) through Burrows-Wheeler Aligner (v0.7.17-r1188) MEM option with default parameters.⁷⁵ Post-alignment processing included the use of Samtools (v1.16.1) for reads sorting and indexing, and further data were processed using Genome Analysis Toolkit (GATK4 v4.2.6.1) (<https://gatk.broadinstitute.org/hc/en-us>).⁶⁴ Key steps using GATK4 included adding read group information by AddOrReplaceGroups, identifying and marking PCR duplicates by MarkDuplicates, and recalibration of base quality scores by BaseRecalibrator. Recalibrated base scores were applied using ApplyBQSR to generate the final analysis-ready BAM file. Read depth of coverage was calculated using Mosdepth,⁶⁵ and post-alignment metrics were generated by GATK4 CollectHsMetrics. Somatic variants calling for single nucleotide variants and small indels were called using MuTect2 in GATK with matched blood. Germline variants commonly found in the general population were filtered out using the af-only-gnomAD VCF file (hg38), which contains allele frequency information from the Genome Aggregation Database (gnomAD) (<https://gnomad.broadinstitute.org/>). The filtered somatic variants were finally annotated using Ensembl Variant Effect Predictor (v106.1) and subsequently converted to the mutation annotation format (MAF) using vcf2maf (v1.6.21).⁶⁶

Copy number variants (CNVs) were identified using CNVkit (v0.9.9).⁶⁷ The analysis was conducted by running the CNVkit batch command with default parameters, using the tumor analysis-ready BAM file as input, along with in-house pooled normal samples as a reference for normalization. Segmentation files and associated copy number calls were used for downstream interpretation of CNVs. R software (v 4.3.2) (<https://www.r-project.org/>) were used for data visualization. Mutational signatures were identified using deconstructSigs (v1.9.0) package.⁶⁸

RNA sequencing data analysis

For RNA-seq data, trimmed reads were aligned using STAR (v2.7.9a) aligner with default parameters for splice-aware mapping.⁶⁹ The resulting aligned reads were processed for transcript-level quantification using RSEM (v1.3.1),⁷⁰ which estimated transcript abundance in terms of transcripts per million (TPM) and expected counts. The transcript quantification results were then utilized for further downstream statistical analysis such as identifying differentially expressed genes (DEGs). Gene Set Variation Analysis (GSVA) and Gene Set Enrichment Analysis (GSEA) were performed to assess gene set level analysis within individual samples and across samples respectively.^{71,76} The clusterProfiler R package was used for pathway enrichment and interpretation, facilitating downstream functional annotation of the results.⁷⁷

GBO-based drug sensitivity testing

GBOs cultured for 1–2 weeks were finely sectioned and treated with Accutase (STEMCELL technologies) to obtain single-cell suspensions. Cells were seeded into 384-well ultra-low attachment plates at 250 cells per well and incubated for 24 h. Subsequently, cells were treated with a range of drug concentrations for six days. Drugs tested included TMZ, regorafenib, lazertinib, olaparib, bevacizumab, and lomustine, with concentrations based on previous studies.^{78–82} Controls included 5 μ M bortezomib as a positive control and 0.5% DMSO and PBS as negative controls. Cell viability was measured on day six post-treatment using the CellTiter-Glo assay (Promega), and ATP levels were quantified using a luminometer (Varioskan LUX Multimode microplate reader, Thermo Fisher Scientific). Viability data were normalized to the average DMSO control values.

The growth curve and number of proliferating cells were measured to confirm the cell viability test result for TMZ, regorafenib, lazertinib. GBOs were dissected into small fragments with diameters ranging 300 to 500 μ m and cultured in 6-well plates for 24 h for stabilization. Subsequently, the fragments were transferred to 96-well plates with 200 μ L of medium supplemented with specific drugs: 100 μ M TMZ, 10 μ M regorafenib, or 10 μ M lazertinib. Media were refreshed every other day. Bright-field images were captured on days 0, 7, 14, and 28. GBO diameters were measured using ImageJ software (imagej.net), and their 3D volumes were calculated to generate growth curves. On day 14, GBOs were transferred to 6-well plates and treated with 10 μ M EdU for 6 h to label proliferating

cells. Proliferating cells were analyzed using the Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 (Thermo Fisher Scientific) according to the manufacturer's protocol.

RNA extraction and quantitative PCR analysis

Total RNA was prepared using TRIzol reagent (Invitrogen), and cDNA was generated using PrimeScript RT Master Mix (Perfect Real Time) (Takara) following the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed with CFX Opus 96 (Bio-Rad). Relative mRNA levels were normalized to β -actin mRNA levels. The primers used for qPCR are described in Table S5.

Orthotopic transplantation of GBOs

Orthotopic transplantation of GBOs was performed as previously described for brain organoid implantation in immunodeficient mice.²⁹ NSG mice were anesthetized with isoflurane, induced at 5%, and maintained at 1%–2% in oxygen. A midline incision (~0.7 cm) was made to expose the bregma, and a burr hole (~0.7 mm in diameter) was drilled at stereotaxic coordinates relative to bregma (AP -0.5 mm, ML $+1.8$ mm, DV -3.5 mm). GBOs were mechanically minced immediately prior to transplantation, suspended in PBS, loaded into a 26G Hamilton syringe, and injected at a rate of $0.2 \mu\text{L}/\text{min}$ to a total volume of $2 \mu\text{L}$. After injection, the needle was left in place for 5–10 min to minimize reflux before slow withdrawal, and the incision was closed with nylon sutures. Postoperatively, mice were kept on a 37°C heating pad until full recovery from anesthesia and received standard analgesia with daily monitoring for signs of distress.

In vivo drug administration

Ten days after xenograft implantation, mice were randomized into treatment groups (5–8 per group). Lazertinib (25 mg/kg; Selleckchem, Cat# S8724) was administered orally once daily for 21 days. Temozolomide (50 mg/kg; Selleckchem, Cat# S1237) was administered by oral gavage on a 5-day-on/2-day-off schedule for 21 days. Lomustine (20 mg/kg; Selleckchem, Cat# S1840) was first dissolved in DMSO and then diluted with PBS to a final concentration of 25% DMSO, and injected intraperitoneally once weekly for 21 days.

For immunohistological analysis, perfused brains were fixed in 4% PFA overnight and cryoprotected in PBS containing 30% sucrose. Serial coronal sections were prepared using a sliding microtome (Leica Microsystems, SM2010R) and stored at -20°C in multiwell plates containing antifreeze solution (30% sucrose, 30% ethylene glycol in 0.1 M PBS). To assess drug sensitivity, IHC staining was performed as described above. Each stained section was compartmentalized into circles with a radius of $200 \mu\text{m}$, and the proliferation index was calculated as the ratio of Ki-67⁺HuNu⁺ cells to total HuNu⁺ cells within each compartment. For each mouse, three sections were analyzed, and the average value was used for quantification.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical significance was assessed using the Spearman correlation test, unpaired t test, multiple unpaired t test, or ordinary one-way ANOVA test. A p -value of <0.05 was considered significant. In the figures, asterisks denote statistical significance ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$). Statistical analyses were performed using GraphPad Prism 10.

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Supplemental information

Patient-derived organoids predict personalized drug response and reveal alternative therapeutic options in glioblastoma

Jisu Park, Meiyu Qiu, Eun Jeong Cho, Sanghee Seo, Ji-Hoon Moon, Minji Kim, Hyemin Mun, Hyounji Yun, Hoewon Park, Arim Lim, Younsoo Kang, Jeong-woo Oh, Heejeong Youk, Seon-Hwan Kim, Yeongbeom Seo, Sae Min Kwon, Kyoung Su Sung, Hyuk-Jin Oh, Kyung Rae Cho, Kyungtae Yoon, Nam-Shik Kim, Se Jin Jang, Kyung Hwan Kim, and Ki-Jun Yoon

Supplementary Figures

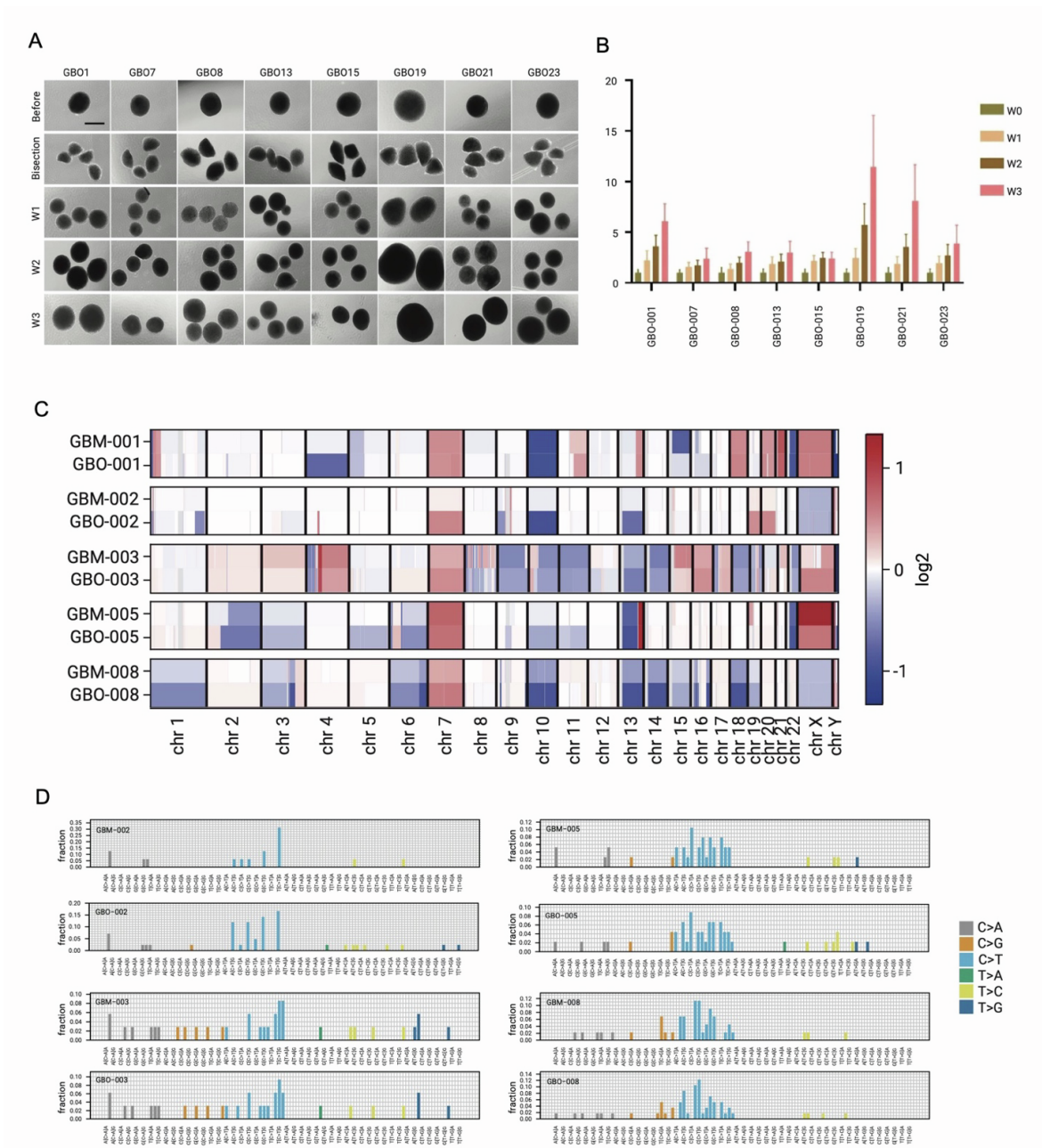


Figure S1. Growth characteristics and genomic landscape of patient-derived GBOs. Related to Figure 1.

- (A) Microscopic images of 8 GBOs after bisection and over time. Scale bar: 500 μ m.
- (B) Growth curves over time of 8 GBOs. The 3D volume of GBO was calculated based on its diameter.
- (C) Copy number variation (CNV) correlation across chromosomes (chr1 to chrY). Heatmap illustrating the correlation of CNVs between GBOs and parental tissues.
- (D) Single nucleotide variant correlation. Bar plot showing the consistency in parental tissues and GBOs.

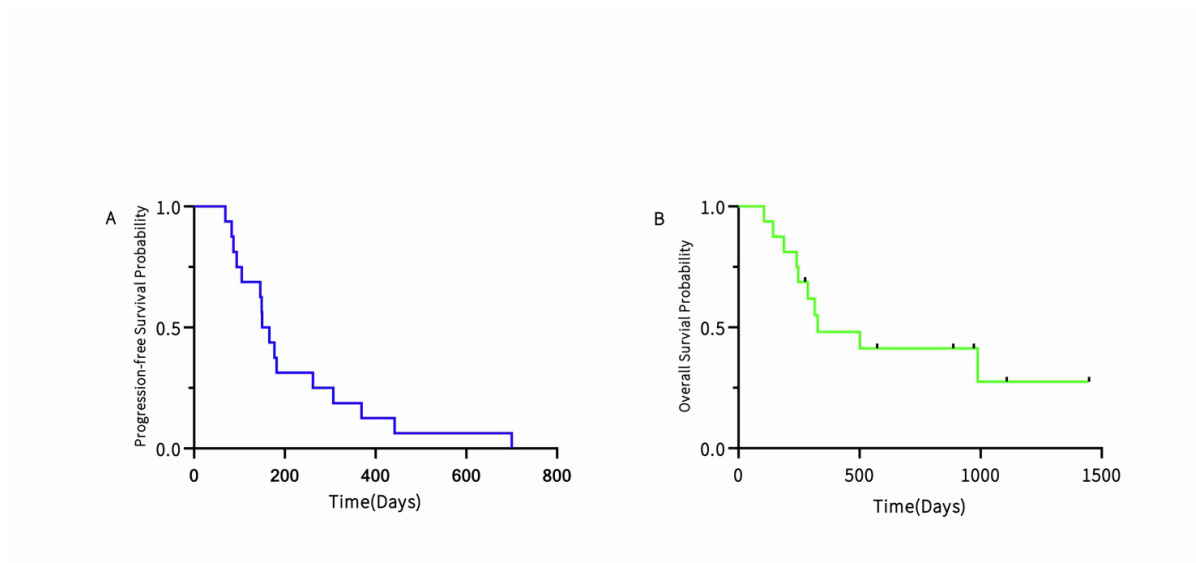


Figure S2. Survival outcomes of the clinical validation cohort. Related to Figure 2.

(A) Kaplan-Meier curve showing PFS of patients in the cohort

(B) Kaplan-Meier curve showing OS of patients in the cohort

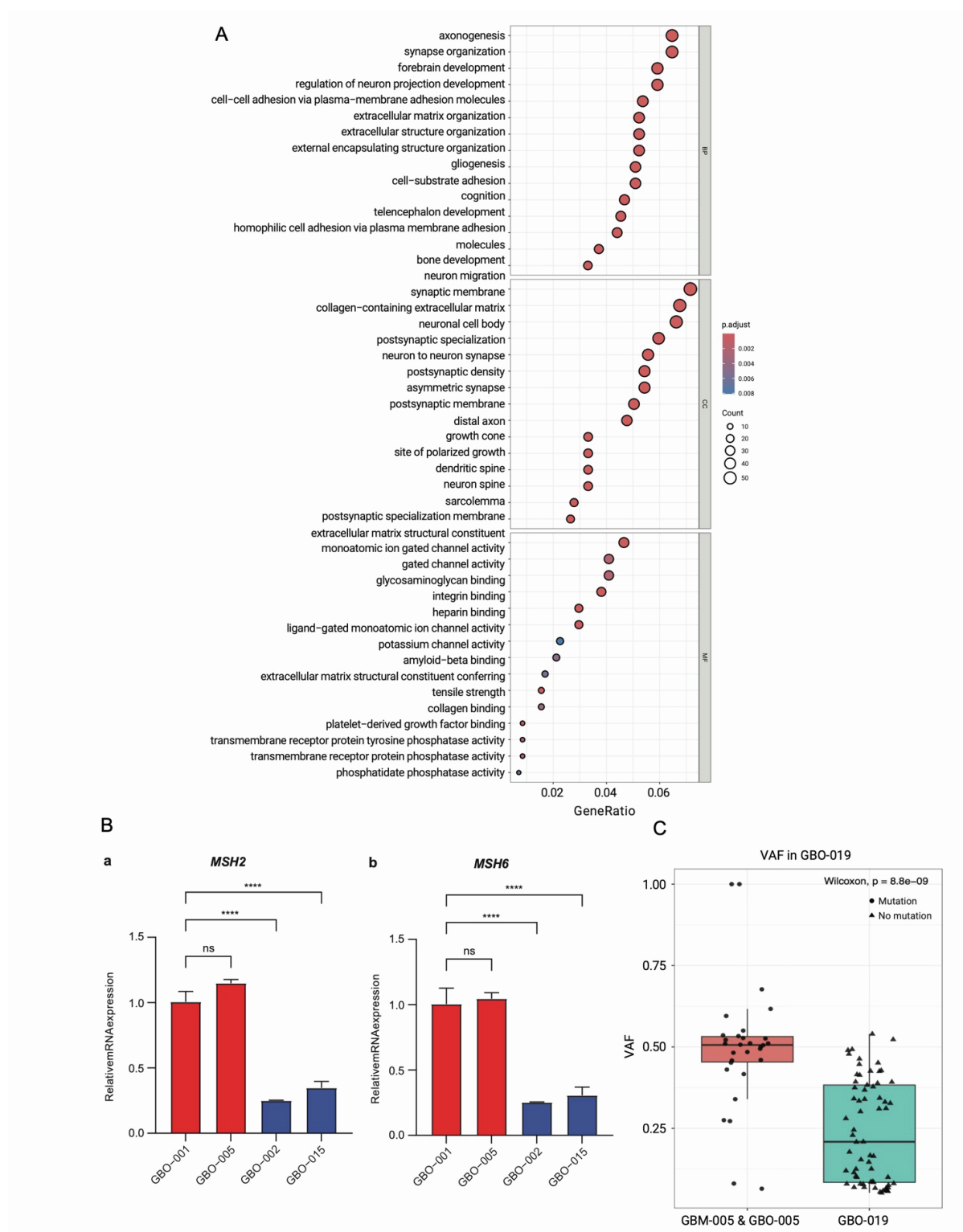


Figure S3. Transcriptomic characterization of TMZ-resistant GBOs. Related to Figure 4.

(A) GO analysis shows that genes associated with synapse organization, axonogenesis, and cell-cell adhesion processes are upregulated in TMZ-resistant GBOs.

(B) qPCR validation of MMR-related gene expression between TMZ-sensitive (Red) and TMZ-resistant (Blue) groups.

Related to Figure 4. Values were normalized to β -Actin and are shown as mean $\pm$ SEM (n=3 technical replicates). Statistics were performed using Dunnett's multiple comparison test against GBO-001. P-values: *MSH2* – GBO-005 (p=0.1728), GBO-002 (p<0.0001), GBO-015 (p<0.0001); *MSH6* – GBO-005 (p=0.8346), GBO-002 (p<0.0001), GBO-015 (p<0.0001). ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

(C) Box plot compares the variant allele frequency (VAF) distribution between GBM-005 & GBO-005 and GBO-019.

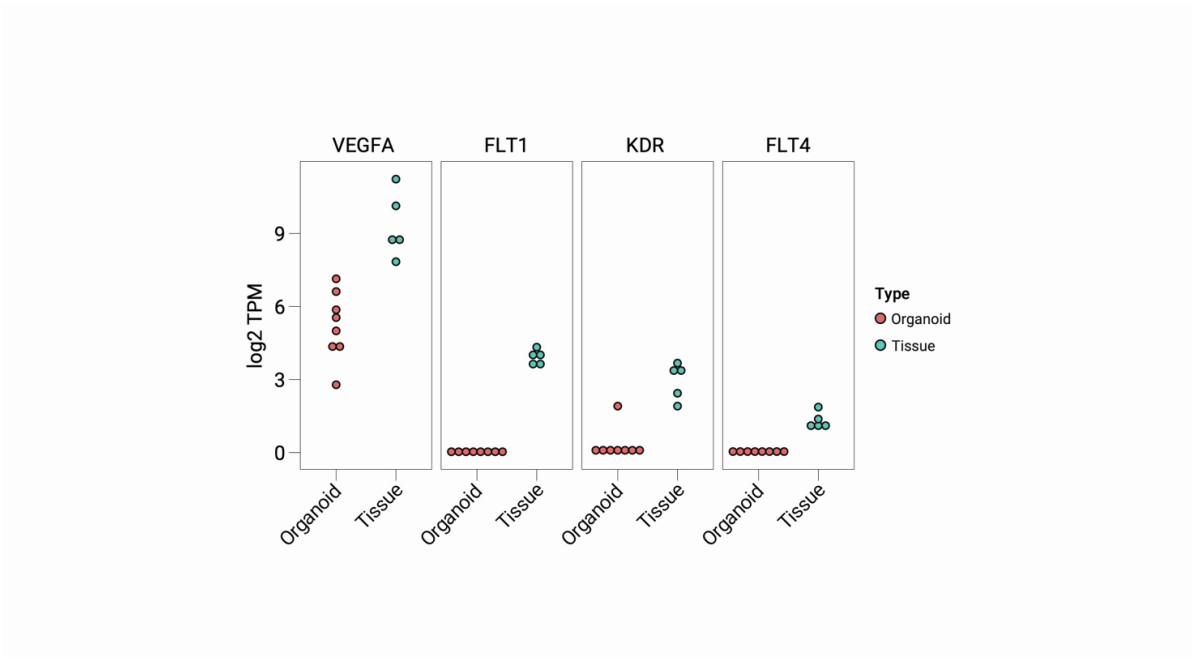


Figure S4. Dot plot showing TPM of VEGF receptors comparing GBOs and parental tissues. Related to Figure 5.

GBO ID*	Age	Clinically recorded gender	GBO Success	Size (cm)	Tumor main location	Initial infiltration	EOR	CCRT**	MGMT (M/UM)	Ki-67 (%)
1	53	F	Yes	4.6	Rt. temporal		CR	Standard	M	40
2	73	M	Yes	6.0	Rt. temporal	LMD	PR	Hypofractionated	M	40
3	81	F	Yes	3.4	Rt. parietal		NTR	Hypofractionated	UM	50
4	63	M	Failure	5.1	Rt. temporo-parietal	LMD	PR	Standard	UM	40
5	57	M	Yes	6.6	Rt. occipital		NTR	Standard	M	30
6	70	F	Yes	5.1	Lt. temporal	MF	STR	Standard	UM	40
7	71	M	Yes	7.0	Lt. temporo-parietal		STR	Not received	UM	50
8	77	M	Yes	5.1	Lt. temporo-parietal		PR	Standard	M	15
9	68	M	Yes	6.1	Rt. temporo-parietal		CR	Standard	UM	10
12	64	F	Failure	3.1	Lt. parietal	GC, MF	PR	Standard	UM	20
13	77	M	Yes	7.5	Lt. temporal	LMD	PR	Not received	M	20
15	69	M	Yes	6.1	Rt. temporal		CR	Standard	M	10
16	65	M	Failure	7.4	Rt. frontal	MF	NTR	Standard	UM	40
18	66	M	Failure	8.2	Lt. temporal	GC, MF	STR	Standard	UM	40
19(5R)	57	M	Yes	6.7	Rt. occipital		CR	Not applicable	M	30
21	67	M	Yes	2.3	Rt. frontal	GC, MF	STR	Standard	UM	20
22	78	M	Failure	4.5	Rt. cerebellum	MF	NTR	Standard	UM	50
23	61	M	Yes	7.1	Rt. temporal		CR	Standard	UM	70
24	60	M	Yes	4.5	Rt. parietal		CR	Standard	M	30
25	72	F	Yes	4.0	Lt. parietal	MF	NTR	Standard	UM	10
26	49	F	Failure	4.2	Lt. fronto-parietal		CR	Standard	UM	5
27(15R)	70	M	Failure	4.0	Rt. temporal		CR	Not applicable	UM	2
28	80	M	Yes	6.7	Lt. temporal		STR	Hypofractionated	UM	30
29	64	M	Failure	5.0	Rt. frontal	LMD	Biopsy	Not received	UM	50
30	57	F	Yes	2.7	Lt. frontal		CR	Standard	UM	20
31	71	F	Yes	4.3	Rt. occipital		CR	Standard	UM	30
32	72	F	Failure	7.3	Lt. temporal	MF	STR	Hypofractionated	M	40
33	80	F	Yes	3.0	Rt. frontal		CR	Hypofractionated	UM	50
34	65	F	Failure	2.5	Lt. cerebellum	GC, MF	PR	Standard	M	5
35	82	M	Failure	4.3	Lt. frontal		Biopsy	Hypofractionated	UM	20

36	74	M	Yes	4.3	Lt. parietal		STR	Standard	M	15
37	60	F	Failure	2.5	Lt. frontal		CR	Standard	M	30
39	69	F	Failure	8.9	Rt. insular	GC, MF	STR	Standard	UM	16
41	73	M	Failure	5.7	Rt. temporal		CR	Standard	UM	20
42(22R)	79	M	Yes	5.3	Rt. cerebellum		NTR	Not applicable	UM	70
43	78	F	Yes	4.1	Lt. frontal		STR	Standard	M	50
44	79	F	Yes	5.9	Rt. temporal		STR	Standard	UM	46
45	60	F	Yes	5.0	Lt. fronto- parietal		STR	Standard	M	5
46	64	M	Yes	3.7	Rt. frontal		STR	Not received	M	10
47	59	F	Yes	4.9	Lt. occipital		CR	Standard	UM	25
48	61	M	Yes	6.7	Lt. frontal	MF	STR	Hypofractionated	M	20
49	69	F	Failure	5.2	Lt. temporo- parietal		CR	Standard	UM	25
50	55	M	Yes	6.4	Lt. frontal	MF	STR	Hypofractionated	UM	30
51	50	M	Yes	10.9	Rt. fronto- temporal	GC, MF	PR	Standard	UM	46
52	57	F	Yes	6.0	Lt. frontal		CR	Standard	M	50
53	69	M	Failure	4.9	Lt. frontal		CR	Not received	M	5
55	37	M	Yes	6.5	Lt. temporal		STR	Standard	UM	20
56	57	M	Yes	4.1	Rt. frontal		GTR	Standard	UM	10
58	79	F	Failure	3.6	Rt. temporal	GC	CR	Hypofractionated	UM	30
60	67	F	Failure	4.0	Lt. fronto- parietal		NTR	Standard	UM	15
61(30R)	58	F	Yes	7.4	Rt. frontal	MF	CR	Not applicable	UM	15
64	65	F	Yes	2.3	Lt. fronto- temporal		NTR	Standard	UM	15
65	63	M	Failure	6.5	Rt. parietal		STR	Standard	UM	15
66	78	M	Failure	6	Rt. temporal		NTR	Standard	M	11
68	82	F	Failure	4.5	Lt. thalamus	MF	Biopsy	Not received	UM	5
69	63	F	Failure	5.4	Lt. occipital		NTR	Standard	M	20
70	47	F	Failure	12.3	Rt. frontal	GC	NTR	Standard	M	30
73	68	M	Yes	1.7	Rt. temporal		STR	Not received	UM	50
74	71	F	Yes	4.2	Lt. frontal		STR	Standard	UM	30

GBO = glioblastoma organoid; EOR = extent of resection; CCRT = concurrent chemoradiation therapy; MGMT = O⁶-methylguanine-DNA methyltransferase; M = methylated; UM = unmethylated; F = female; M = male; LMD = leptomeningeal disease; MF = multifocal enhancement; GC = gliomatosis cerebri; CR = complete resection; PR =

partial resection; NTR = near-total resection; STR = subtotal resection

*GBO 19 and 27 were the recurrent samples of GBO 5 and 15, respectively; GBO 9 was successfully generated, but lost due to a human error.

**Standard CCRT consisted of 60 Gy in 30 fractions with daily temozolomide (75 mg/m²) administration, while hypofractionated CCRT was 45 Gy in 15 fractions, determined according to the performance status of patients.

Table S1. Patient characteristics and outcomes of glioblastoma organoid establishment. Related to Figure 1 and STAR Methods.

Variables	Univariate analysis			Multivariate analysis		
	OR	95% CI	p-value	OR	95% CI	p-value
Age	1.020	0.965–1.081	0.489	1.032	0.930–1.187	0.331
Clinically recorded female	1.527	0.533–4.441	0.431	1.512	0.479–4.880	0.481
Extensive infiltration*	2.750	0.913–8.606	0.075	3.528	1.062–13.150	0.046
MGMT methylation	0.689	0.217–2.057	0.509	0.628	0.177–2.089	0.455
Lower Ki-67	1.030	0.996–1.068	0.100	1.042	1.003–1.090	0.047

OR = odds ratio; CI = confidence interval; MGMT = O⁶-methylguanine-DNA methyltransferase

*Extensive infiltration referred to the initial presentation of the tumor on radiological imaging with features such as multifocal enhancement, gliomatosis cerebri, or leptomeningeal disease.

Table S2. Risk factor analysis for failure to form glioblastoma organoids; A total of 59 GBM samples were included in the analysis. Related to Figure 1 and STAR Methods.

Subgroup*		GBO ID	Age	Clinically recorded gender	EOR	IC50 (μM)	AUC	PFS (days)	Salvage treatment	Death	OS (days)
TMZ sensitivity	MGMT Status										
Sensitive	Methylated	1	53	F	CR	23	139	369	GKS, BEV+IRI	Alive	1448
		5	57	M	NTR	5	109	307	Re-op, BEV+IRI	Death	988
		8	77	M	PR	41	196	262	None	Death	315
		24	60	M	CR	40	179	700	Re-op, Re-TMZ	Alive	972
		52	57	F	CR	31	229	442	BEV+IRI	Alive	573
Resistant	Methylated	2	73	M	PR	548	439	86	None	Death	187
		15	69	M	CR	>1000	354	94	Re-op, BEV+IRI	Alive	1108
		19(5R)	57	M	CR	594	545	N/A	N/A	Death	988
		48	61	M	STR	>1000	451	182	None	Death	327
Resistant	Unmethylated	3	81	F	NTR	>1000	427	83	None	Death	143
		6	70	F	STR	552	453	105	None	Death	425
		21	67	M	STR	600	462	146	BEV+IRI	Death	501
		23	61	M	CR	613	391	150	BEV+IRI	Alive	887
		25	72	F	NTR	>1000	387	149	None	Death	286
		28	80	M	STR	653	514	69	None	Death	247
		50	55	M	STR	714	494	166	None	Death	240
		61(30R)	57	F	NTR	>1000	359	N/A	BEV+IRI	Death	581
		74	71	F	STR	>1000	547	177	BEV+IRI	Alive	275

TMZ = temozolomide; MGMT = O⁶-methylguanine-DNA methyltransferase; GBO = glioblastoma organoid; EOR = extent of resection; IC50 = half maximal inhibitory concentration; PFS = progression-free survival; OS = overall survival; F = female; M = male; CR = complete resection; NTR = near-total resection; PR = partial resection; STR = subtotal resection; N/A = not applicable; GKS = Gamma-Knife radiosurgery; BEV+IRI = bevacizumab combined with irinotecan chemotherapy

*There was no GBO subgroup that is simultaneously TMZ-sensitive and MGMT unmethylated.

Table S4. Subgrouping according to the temozolomide sensitivity test and MGMT methylation status; Clinical characteristics and temozolomide sensitivity results of clinical correlation cohort. Related to Figure 2.

Primer	5' - 3'
<i>hACTB</i> forward	CATGTACGTTGCTATCCAGGC
<i>hACTB</i> reverse	CTCCTTAATGTCACGCACGAT
<i>hMAPT</i> forward	CCTGGTAGCTCAGACCCTCT
<i>hMAPT</i> reverse	CGTTTTACCATCAGCCCCCT
<i>hGPM6A</i> forward	GAGTCACGGCTTTCACCTCA
<i>hGPM6A</i> reverse	ACTGACGAAGGTCCAAGCAG
<i>hNCAM1</i> forward	CAACCTGTGTGGAAGCCG
<i>hNCAM1</i> reverse	CATGGTTTGGGGTCCTCTCC
<i>hPTPRZ1</i> forward	TGGATCAGGCAGAGAGAGCT
<i>hPTPRZ1</i> reverse	GGGACCCTGTGACATCACTG
<i>hMSH2</i> forward	TTTACCCGGAGGAGAGACTGC
<i>hMSH2</i> forward	TGCTCTCCCTTTTGCCTTTC
<i>hMSH6</i> forward	AGAGCAATGCAACGTGCAGA
<i>hMSH6</i> forward	TTTGGCGGCTACTTCGCCTA

Table S5. Primer information for qPCR. Related to STAR Methods.