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Review article

Non-animal glioblastoma models for personalized treatment

Alja Zottel *, Ivana Jovčevska, Neja Šamec

Centre for Functional Genomics and Bio-Chips, Institute of Biochemistry and Molecular Genetics, Faculty of Medicine, University of Ljubljana, Zaloška cesta 4, 1000, Ljubljana, Slovenia

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ABSTRACT

Glioblastoma is an extremely lethal cancer characterized by great heterogeneity at different molecular and cellular levels. As a result, treatment options have moved far from systemic and universal therapies toward targeted treatments and personalized medicine. However, for successful translation from preclinical studies to clinical trials, experiments must be performed on reliable disease models. Numerous experimental models have been developed for glioblastoma, ranging from simple 2D cell cultures to study the nature of the disease to complex 3D models such as neurospheres, organoids, tissue-slice cultures, bioprinted models, and tumor on chip, as perfect prototypes to evaluate the therapeutic potential of different drugs. The presence of multiple research models is consistent with the complexity and molecular diversity of glioblastoma. The advantage of such models is the recapitulation of the tumor environment, and in some cases the preservation of immune system components as well as the creation of simple vessels. There are also two case studies translating *in vitro* studies on glioblastoma organoids to patients as well as four ongoing clinical trials using glioblastoma models, indicating high clinical potential of glioblastoma models.

1. Introduction

Glioblastoma is the most common primary brain tumour and is an extremely lethal cancer with the 5-year survival rate less than 10 % [1]. It is a rare cancer with an incidence of approximately 3/100 000 people in the United States [1]. About 5 % of gliomas are familial and have a genetic predisposition, such as Li-Fraumeni syndrome and Lynch syndrome [1]. Symptoms of the disease can be vague, making early diagnosis difficult. Standard therapy consists of surgical removal of the tumor, radiotherapy and chemotherapy with temozolomide [2], while second-line therapies include bevacizumab, lomoustine, regorafenib, vincristine and alternating treating field [3–7]. Despite advances in treatment, the majority of patients die within 18 months of diagnosis [8]. The reasons for treatment failure are multifactorial. The brain is surrounded by a blood-brain barrier that prevents penetration of the majority small molecules [9]. Glioblastoma is also a highly infiltrative tumor, leading to recurrence [10]. One of the main causes for treatment failure is exceptionally high heterogeneity, which pushes the treatment towards personalized medicine rather than choosing universal drug.

In this review we describe glioblastoma heterogeneity and its relation to treatment options. As a core of this paper, we present several experimental glioblastoma models and highlight their potential for clinical implementation. Finally, we discuss the different approaches for studying immunotherapy in glioblastoma models and present cases studies and clinical trials including glioblastoma models.

* Corresponding author.
E-mail address: alja.zottel@mf.uni-lj.si (A. Zottel).

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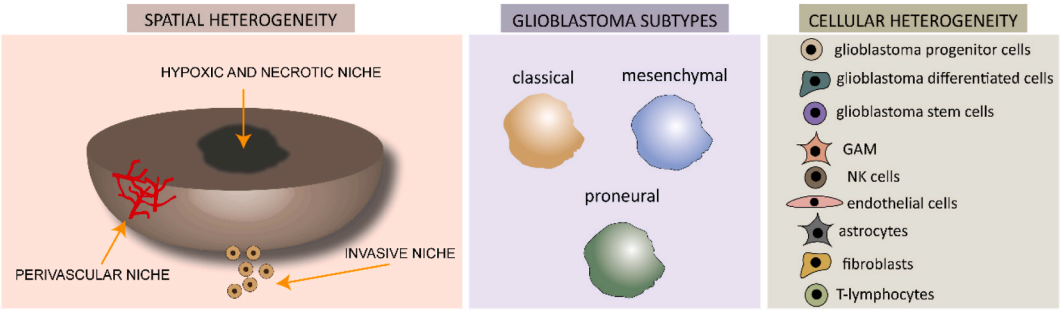

Fig. 1. Glioblastoma heterogeneity. The figure represents several levels of glioblastoma heterogeneity, including spatial, glioblastoma subtypes and cellular heterogeneity. GAM – glioma-associated macrophages, NK cells – natural-killer cells.

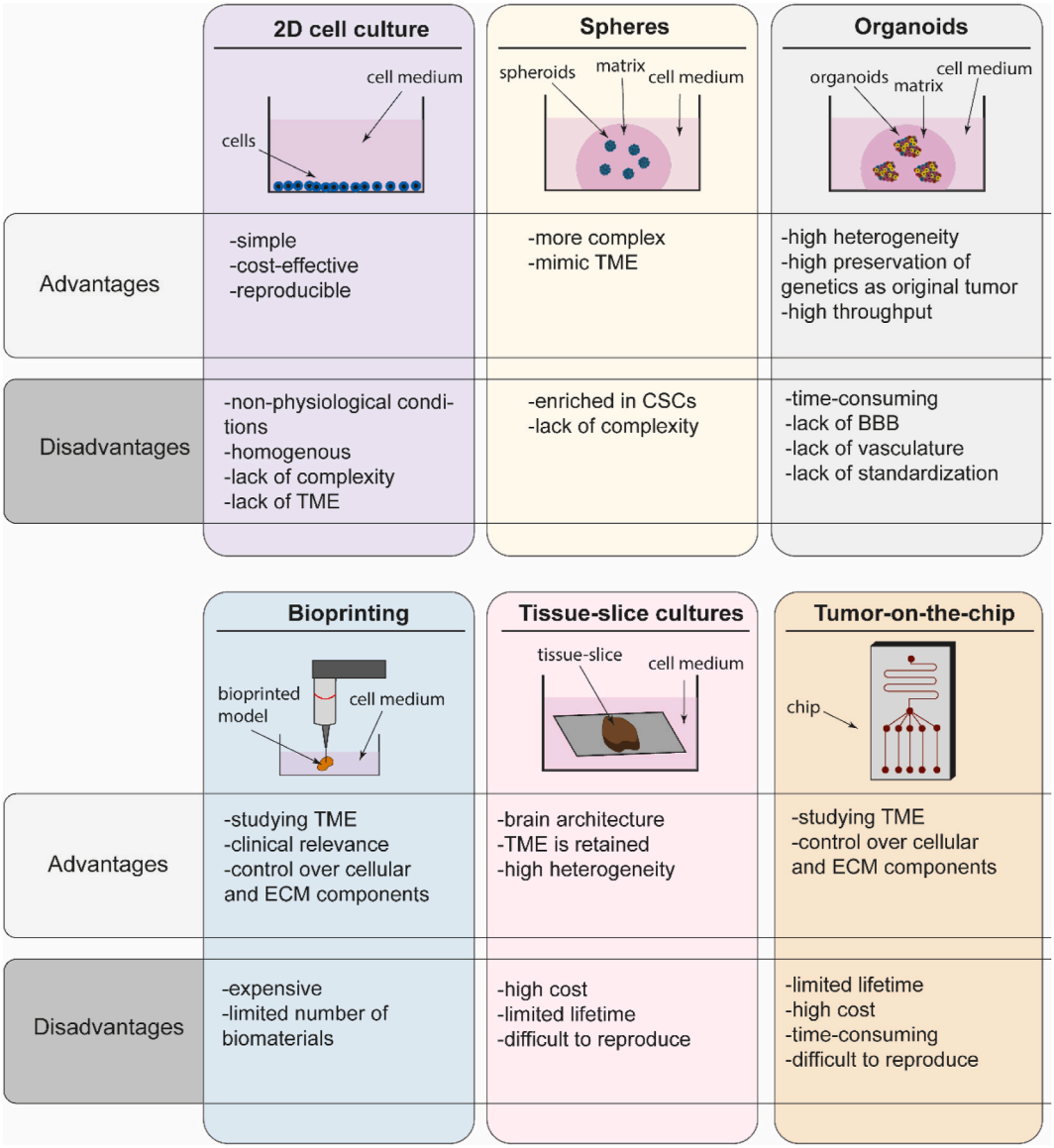

Fig. 2. Non-animal glioblastoma models and their advantages and disadvantages. The figure represents several models, including 2D cell culture, spheres, organoids, bioprinting, tissue-slice cultures and tumor-on-the-chip. TME – tumor microenvironment, CSCs – cancer stem cells, BBB – blood-brain barrier, ECM – extracellular-matrix.

2. Glioblastoma heterogeneity

Glioblastoma is a remarkably heterogeneous tumor at several levels (Fig. 1). Based on transcription analysis, Verhaak et al., 2010 classified glioblastoma into four subtypes based on gene expression: classical, mesenchymal, proneural, and neural. The proneural type is more common in younger patients and has the same profile as secondary glioblastoma [11]. In 2017 Behnan et al. similarly subdivided the tumors into classical, mesenchymal and proneural subtypes, with the previously described neural subtype belonging to the proneural subtype [12]. Tumor subtypes are not fixed, as more than one subtype may be present in the same tumor and may change in case of recurrence, which also shifts the phenotype toward selection of resistant clones [13,14]. The recurrence may also cause genetic alterations. It has been observed that mutations in *EGFR*, *PDGFRA* and *PTEN* are present in both tumors, whereas mutations in *TP53*, *EGFR* and *CDKN2A* were lost in the recurrent tumor and mutations in *LTBP4*, *PRDM2*, *IGF1R*, and *MSH6* were detected only in the recurrent tumor [15]. However, other studies suggest that genetic changes after treatment are very limited and are more a consequence of the presence of tumor cells after surgery [16].

The tumor is composed of various cells, including the most malignant, glioblastoma stem cells (GSCs). They are self-renewing, generate both stem and functional cells, proliferate slowly and are capable of forming a tumor when transplanted into an animal [17]. GSCs express markers of neural stem cells and progenitor cells, such as Sox2, Nanog, Oligo2, Myc, Musashi, BMI1, Nestin, and ID1 [18]. With the normal stem cells, GSCs share several signaling pathways, such as Wnt/beta-catenin, SHH and Notch [19–21]. They represent only a small fraction of cells (0.01–2% of the total tumor mass), are resistant to chemotherapy and radiotherapy and have been implicated in tumor recurrence [13,22]. Several studies show that the population of GSCs in the tumor is not uniform but highly heterogeneous [23]. Recent studies suggest that hierarchical cancer stem cell theory does not fully explain tumor biology, because cells lacking stem cell markers are capable of self-renewal, unlimited proliferation, and tumorigenicity. Cancer stem cells also have a rather high plasticity and can exist in different states depending on the tumor environment [16]. GSCs are closely associated with tumor microenvironment and are found in various tumor niches, including the perivascular niche, where they stimulate vessel growth, hypoxic and necrotic niche, which stimulates quiescence, and the invasive niche, which is located on the tumor rim and stimulates cell migration [24].

3. Non-animal glioblastoma experimental models

Cancer models are essential for understanding the development and behavior of human disease. This involves either understanding the fundamental processes that occur within the cell and lead to tumor development, or modeling complex processes between the tumor and the tumor microenvironment (TME) that support tumor growth. Appropriate experimental models that accurately mimic the tumor can be used to evaluate different therapeutic strategies. Several cancer models are available for glioblastoma, most of which are discussed below and presented in Fig. 2. In recent years, the emphasis in drug testing has shifted from animal models to nonanimal models. A major impetus for the development of cancer models has been the removal of the requirement for animal testing by the FDA in 2022 [25]. This will further encourage scientists to develop non-animal models. Therefore, in this paper we focus only on non-animal models.

3.1. 2D models

Glioblastoma is a complex tumor that is part of a multilayered TME composed of extracellular matrix (ECM) components and infiltrated cancer-associated cells [26]. A drawback in glioblastoma research is the lack of suitable *in vitro* model systems. For a long time glioblastoma research was based on the use of immortalized 2D monolayer cell cultures grown in serum-containing conditions. 2D models are used in research because of their simplicity and cost-effectiveness [27]. 2D cultures can be commercially available i.e. derived clonally from patient tumors, the best known being U87MG, U251MG, A172, and LN-229 [28], or primary i.e. early-passage patient-derived cells established from fresh or cryopreserved tumor specimen under appropriate conditions [29]. They provide experimental replicability and reproducibility, and also allow comparison of results between different laboratories. However, they have important limitations. First, most commonly used 2D cultures do not mimic the complexity and *in vivo* tumor properties [28] and are often cultured under nonphysiological conditions [30]. For example, 2D cultures are cultured in an environment with 20 % oxygen, while glioblastoma cells grow *in vivo* at an oxygen tension of 0.1 %–10 % [31]. Another disadvantage are genetic and epigenetic alterations due to the lack of interactions between glioblastoma cells and TME [32]. In addition, prolonged culturing in the presence of serum can significantly alter cell properties [33]. This is because prolonged culturing selects the cells with the highest proliferative potential and reduces the cellular heterogeneity of the parent tumor [34]. During this process, quiescent glioblastoma stem cells, responsible for radiation and chemotherapy resistance, may be lost over time, limiting ability to study them [35]. When glioblastoma stem cells are cultured with serum, they lose their stemness properties. In addition, they are homogeneous and are not a realistic image of the actual tumor [36]. This problem can be avoided by using patient-derived cell lines maintained in the absence of serum and at low passage to preserve original phenotype and genotype. Patient-derived cell lines are a better preclinical model as they maintain the genetic heterogeneity and characteristic of the original tumor. To avoid genetic and transcriptomic alterations, these cells should not be grown in culture for more than 20 passages [28]. Patient-derived cell lines can be grown in either 2D (monolayer) or 3D (spheroids) cultures. Recently, efforts have been made to store such patient-derived models in a biobank and make them available to the broader research community [37].

Nonetheless, 2D *in vitro* systems are a practical and rapid model to obtain information about important cell mechanisms and functions, to investigate a specific cell feature of interest, to identify novel biomarkers, and to obtain preliminary results. However,

these findings need to be confirmed in more appropriate and advanced models. Some examples of the use of 2D models in drug validation are presented below.

Aljohani et al. analysed the tyrosine kinase inhibitor resistance in glioblastoma cell lines [38]. They selected U87 cell line clones that were resistant to gefitinib, inhibitor of tyrosine kinase, and performed RNAseq to determine the differences in signalling pathways between resistant and non-resistant cells. Several upregulated genes were discovered, mainly tyrosine kinase receptors *ROS1*, *DDR1* and *PDGFRA*. Overexpression of ROS1 was confirmed also on protein level. Resistant clones were then treated with gefitinib in combination with highly effective ROS1 inhibitor, which resulted in substantial cell death.

Studies have also been performed with patient-derived cells, which are more similar to the tumor compared with cell lines. Akgül et al. developed a patient-derived glioblastoma cell line and performed single-cell RNAseq and DNAseq to determine the subtypes and select clones for further analysis [39]. Based on analysis of somatic mutations, copy number status and gene expression, drugs targeting AURKs, CDK4/6, EGFR/mTOR, PDGFR, PI3K, or WNT were selected. Individual clones were then treated with all inhibitors and the drug efficacy was largely consistent with genetic analysis. Two of the drugs tested, vistusertib (anti-mTOR) and buparlisib (anti-PI3K) were highly effective in all clones, whereas afatinib (anti-EGFR) was effective in most clones. Verploegh et al. analysed the resistance mechanisms to bone morphogenetic protein 4 (BMP4) in patient-derived cells [40]. Cells with high and low BMP4-sensitivity were selected for analysis. Both cell types were then treated with BMP4, and single-cell RNAseq was performed. The results showed that OLIG1/2 was a potential predictive biomarker for BMP4 therapeutic success. In addition, the ribosomal translation genes *RPL27A* and *RPS27* were suggested as potential biomarkers for early response to BMP4. BMP4 is a member of the transforming growth factor beta family and plays a critical role in central nervous system development. In adults, it is responsible for balance between neurogenesis and differentiation [41]. In glioblastoma, BMP4 decreases glioblastoma stem cell proliferation and sensitizes to classical therapy [42]. It has also been proposed as a potential therapeutic agent and entered Phase 1 clinical trial for the treatment of patients with progressive and/or multiple recurrent glioblastoma, indicating the importance of patient stratification in terms of the susceptibility to BMP4 [43].

3.2. 3D models

3.2.1. Neurospheres

A better alternative to 2D cell culture are 3D *in vitro* models that can mimic tissue-like features and interactions of the TME. Glioblastoma stem cells are considered the first 3D glioblastoma model because they can be either grown in 2D or 3D in the form of neurospheres [35]. The downside of this model is the necrotic core, which limits the size of neurospheres to 300 μm before they need to be dissociated, and the loss of contact with ECM which does not represent *in vivo* behavior as it is. To mimic the TME, glioblastoma cells are usually cultured in hydrogel coated 3D scaffolds in the presence of various cell types, soluble and ECM signaling factors [34]. Tumor spheres or in the case of gliomas neurospheres, are cell proliferations derived from primary cells of glioblastoma patients and are capable of retaining the characteristics of cancer stem cells, as well as resembling interactions, oxygen and tumor gradient [32]. Neurospheres can be grown in suspension or embedded in gels to simulate cell-ECM interactions [44]. Spheroids exhibit a hypoxic core and necrotic regions and are therefore a more realistic model of glioblastoma itself. For neurosphere maintenance it is important that the cells are grown in serum-free conditions, so they do not lose the properties of the primary tumor [34]. With long-term expansion these cells can also be subject to genetic drift and clonal selection. A drawback of these models is the limited number of cells studied (tissue-derived cancer cells, circulating cancer cells or established cell lines) or the focus on a specific property of the *in vivo* system. For example, Musah-Eroje and Watson established a 3D *in vitro* model system of glioblastoma in which they show that TME factors can influence cell biology. Compared with the corresponding 2D cultures, the 3D neurosphere system showed higher expression of stem cell marker CD133 [44]. In addition, Diao et al. developed a 3D *in vitro* model that mimics the brain tissue [45]. Four neuroepithelial glioblastoma cell lines (LN229, SNB19, U251, and U87) were cultured in hollow 3D micro-chambers, allowing investigation of the invasive properties of glioblastoma. The presence of natural hydrogel, specifically a collagen gel the model a natural-like system that allows cells to attach, proliferate and invade, just as they do *in vivo*. Even though they are better than 2D monolayer cultures, neurospheres also have their limitations. For example, they are enriched with stem cells which are not abundant in actual tumors [46].

As an advanced model Ye et al. developed human patient-derived spheres and xenografts which were tested with various MDM2-p53 inhibitors. RNAseq and genetic analysis revealed that the most sensitive organoids had the highest MDM2 expression and were also p53 wild type. The results suggest that the RNAseq could be used to determine the patient susceptibility towards MDM2-p53 inhibitors [47].

Ratliff et al. developed spheroids from recurrent glioblastoma tissue. The spheroids were treated with the panel of drugs approved by FDA. The results showed that the tumors responded differently to the drugs and that some organoids were resistant to all drugs tested. This method has high clinical potential because results were obtained as early as 15 days after patient surgery [48]. Darrigues et al. developed cell line spheroids and organoids from glioblastoma tissues. The models were screened with 22 anti-invasive compounds targeting NF-kB, GSK-3-B, COX-2, and tubulin [26]. The results showed that spheroids responded very differently to the drugs, reflecting high heterogeneity of the tumor. Jermakowicz et al. developed glioblastoma organoids which were then treated with a novel bromodomain and extraterminal domain inhibitor, that penetrates the brain. The results showed that the inhibitor significantly decreased proliferation and invasion [49]. Bayat et al. analysed the effect of anti-angiogenic drug avarstatin when U87 spheroids were cultured with human umbilical vein endothelial cells in fibrin gel. After 1–2 days, HUVEC formed short, narrow cord-like structures. When co-culture was treated with avarstatin, the tube network was disrupted, with fewer connections and shorter tube length. Detailed analysis showed, that avarstatin decreased proliferation of HUVEC [50].

3.2.2. Organoids

A suitable 3D *in vivo* model are organoids, which can be directly derived from patient tissue [51] or developed from the cell, either pluripotent (embryonic or induced pluripotent) [33] or organ-specific adult [32] stem cells [52]. Organoids are a suitable model for the organ physiology and histology. Using glioblastoma stem cells from patients Linkous et al. established GLICO (cerebral organoid glioma) i.e. they grew an infiltrative glioblastoma in a human cerebral organoid model [53]. Using immunofluorescence microscopy the authors confirmed tumor formation within the organoids. They also confirmed infiltration of glioblastoma stem cells into surrounding tissues, consistent with the nature of glioblastoma in patients. Although very promise for modeling glioblastoma, Linkous et al. believe that their GLICO model is open for further improvement such as the introduction of vasculature or immunological niche. Jacob et al. reported the generation of patient-derived glioblastoma organoids that recapitulate inter- and intratumoral heterogeneity and preserve the key molecular features of their original tumors [51]. The authors performed histologic and morphologic analysis to determine similarity of the organoids to the original tumors; RNAseq to determine the gene expression profile of the generated organoids and demonstrate that the organoids retained the gene expression signatures of their original tumors; exome sequencing to examine genomic alterations and demonstrate that organoids retain the genomic alterations found in the parental tumors; and single-cell RNAseq to show that organoids maintain different cell populations and their specific gene expression profiles. Overall, they conclude that intra-tumor heterogeneity is maintained in organoids just as it is in the original tumor.

A major disadvantage of organoids is lack of immune cells, blood-brain barrier functions and vasculature. A limitation of organoid models is also the absence of endothelial cells which are preferred for invasion, and microglial cells that mediate inflammatory response. These problems can be overcome by preparing organoid co-cultures with endothelial or mesenchymal progenitors, or hematopoietic progenitors, respectively [11]. However, in cases where interactions between specific cells are to be identified, 3D models are more complex than necessary because they are affected by the tumor microenvironment [26]. Below we present some examples of the use of organoids in drug testing for glioblastoma.

Wei et al. investigated the effect of anti-HSP90 drug gamitrinib in glioblastoma cells, neurospheres and patient-derived organoids [54]. They found that gamitrinib inhibited cell proliferation and induced apoptosis in all glioblastoma models. Lenin et al. [55] provided an overview of the potential glioblastoma drugs based on several criteria: currently in phase 2–4 in clinical trials, FDA approved for medical condition other than glioblastoma or prospective inhibitors based on literature, that are not approved by FDA or in clinical trials. The drugs were first tested on two primary glioblastoma cells. The most effective drugs were then tested on glioblastoma organoids, that had been pre-treated with temozolomide and radiation. The compounds were more effective in pre-treated organoids than in untreated ones.

Organoids are an excellent model for studying tumor biology but also for evaluating treatment response in glioblastoma studies. One challenge that remains to be addressed is reducing the diversity of organoids i.e. standardization and reproducibility, while maintaining tumor complexity and heterogeneity. Also, the lack of endothelial cells and vasculature currently limits their utility. In addition, inclusion of microenvironment conditions such as inflammatory and immune responses should also be considered.

3.2.3. Tissue-slice cultures

The first tissue-slice cultures were developed in 1950s by Harford et al. [56]. The cultures are prepared from surgically resected tissue that is cut into cylindrical or cuboid pieces. These are then sliced within 6 h of surgery. Slices are then placed in the incubator where they can be cultured for several days [57]. Such cultures maximally preserve characteristics of original tumor and can be cultured for at least 10 days, while the immune cells are preserved for at least 8 day [57]. Generally, slices are 250–500 μm thick, to avoid brittleness and allow adequate oxygen and nutrients supply [57]. Microdissected brain regions are cultured on a semipermeable membrane in a cell culture insert and are exposed to serum-containing medium [58]. It is recommended that experiments not be performed within 24 h because of increased metabolic activity as a result of stress [59]. Tissue-slice cultures contain different cell layers and parts of the TME [28]. Below we present some examples of using tissue-slice culture for drug testing in glioblastoma.

Marques-Torrejon et al. reported establishment of human whole brain slices and allow the study of glioblastoma stem cell actions [58]. Such models also enable studying cell-migration. Ren et al. developed coculture of brain slices and glioma cells, in which they tracked glioma cell migration. This allowed monitoring of cell invasion, migration rate, and also depth of invasion. Moreover, individual cells could also be monitored. The co-cultures were then treated with a Rac1 inhibitor, which significantly decreased invasion [60]. Merz et al. developed glioblastoma tissue-slice cultures which were treated by temozolomide and irradiation. The slices preserved histopathological properties for at least 16 days. As previously observed, the tumors differed significantly in their susceptibility to temozolomide [61]. Zhao et al. developed tissue slice cultures derived from six glioblastoma patients and performed scRNAseq to opt for the most optimal drug. The slices were treated with etoposide, panobinostat, RO492997, tazemetostat, ispinesib, and Ana-12. After treatment, single-cell RNAseq was performed to analyse the response to each drug, particularly the response of tumoral and non-tumoral cells. Etoposide strongly downregulated cell cycle genes in proliferating cells whereas it had no effect on non-tumoral cells. Panobinostat also affected gene expression in tumoral cells and remodelled myeloid cells in the microenvironment. This type of analysis may also be clinically feasible, because it can be performed within a week of surgical resection [62].

3.2.4. Bioprinting

Bioprinted models are layers of biomaterials composed of living cells of different types [34]. Due to wide range of materials, different viscosities and cell densities, 3D-bioprinted models are a better model for studying TME [63]. In GBM, bioprinted 3D models are mostly constructed by extrusion method [64]. An example of suitable models for studying cellular interactions and therapeutics are 3D-printed mini-brains [65]. Heinrich et al. bio-printed a miniaturized model of a brain with glioblastoma. To test crosstalk between tumor cells and macrophages, the authors examined the expression profiles of genes commonly expressed in glioblastoma. Compared

with 2D models, the 3D models showed increased expression of ECM-remodeling enzymes and phenotypic markers of glioblastoma-associated macrophages (GAM). In addition, the mini-brains exhibited increased expression of EMT biomarkers. To confirm the importance of the mini-brain in glioblastoma, the authors performed transcriptomic analysis of publicly available data. They detected overexpression of eight genes (*FGF2*, *IL-1β*, *MMP2*, *SPP1*, *CCL2*, *CHI3L1*, *MMP9* and *GPNMB*) in patient samples, confirming the clinical relevance of their model. They also tested three different drugs in the 3D models, carmustine and two immunomodulatory drugs, Stat6 inhibitor and CSF1 receptor inhibitor. They observed that IC_{50} of drugs was significantly higher in 3D cultures compared to 2D cultures, which may be due to poor diffusion. They also treated drugs in 3D monoculture and co-culture with macrophages. BCNU had higher effect in co-culture 3D model, probably due to higher proliferation rate compared to monoculture 3D model. In another study, Neufeld et al. [63] developed a 3D-bioprinted glioblastoma model as a platform for drug screening and prediction of treatment response. The model consisted of tumor and vascular tissue, and was able to mimic cellular heterogeneity and cell-cell interactions of *in vivo* grown tumors. In general, 3D-bioprinted models were considered better drug-discovery and screening platform. They could be used either as an adjunct to 2D and 3D *in vitro* studies or as an alternative to *in vivo* mouse models. In another study, Tang et al. [66] developed bioprinted glioblastoma model composed of glioblastoma stem cells, astrocytes, neural precursor cells and macrophages in hyaluronic-acid rich hydrogel. Based on genetic profile and drug-response prediction, the models with or without macrophages were treated with three compounds, abiretarone, vemurafenib and ifosfamide. Interestingly, the sensitivity of drugs differed between the two models.

For glioblastoma research, it is best to have a model that retains its *in vivo* properties but is also simple enough to manipulate for various research purposes. 3D models are undoubtedly the systems that more closely resemble actual human disease, including glioblastoma, compared to 2D. Such systems allow researchers to study the dynamic interactions between glioblastoma cells and the other cells in the TME, which contribute to the heterogeneity, plasticity and development of the tumor. One of the advantages of 3D-printed models is their clinical relevance, as they can replicate the delicate actual system. 3D models will allow us to understand the role of individual cells in the tumor in relation to the immune environment, which will lead to the development of immune-based therapies. 3D models are also useful for drug discovery studies because they show the disease in all its complexity. Therapeutics can be tested on such realistic systems, which will ultimately reduce the need to use laboratory animals and accelerate the development of precision oncology and individualized therapy.

3.2.5. Tumor on the chip

Organ on the chip is a microfluidic device usually fabricated using polydimethylsiloxane. It provides dynamic culturing of cells using micro-pump, which administers nutrients and discharges waste. The chip is composed of four crucial components: microfluidic, sample, stimulation or drug delivery and sensing. Usually, biocompatible hydrogel is added to the chip [67]. Organ on the chip also enables forming vasculature and studying interactions between cancer and other organ [68]. These models are suitable for tracking dynamic cell behavior, as well as imaging and tracking cell responses in regard to the microanatomical location of the tumor. They are convenient for studying cell migration and invasion cell properties in the real TME [69]. Although they provide access to brain architecture, reduce the need for experimental animals and accelerate experimental throughput, the drawback of these models is their limited lifetime of approximately 3 weeks, high cost, time consumption and inability for reproducibility. Currently, they are not an equivalent alternative to animal models, as they cannot recapitulate different biological and biochemical processes. They are also difficult to handle and are therefore challenging for high throughput analyses [70]. Below we present two examples of the use of tumor on chip in drug testing for glioblastoma.

Akay et al. developed 3D spheroids from primary glioblastoma cells and cultured them on the chip. The chip consisted of a hydrogel solution of 20 % poly(ethylene glycol) diacrylate in phosphate-buffered saline. It had two inlets that formed a gradient of two different drugs, thus allowing the study of the combined effect of two different drugs. Models of three different glioblastoma patients were treated with temozolomide and/or bevacizumab. The response of individual patients varied, but in general, the combination of the two drugs had a better effect than single therapy. The chip has the potential to be used in clinics because it is rapid, simple, and inexpensive [71]. Similarly, Fan et al. developed a glioblastoma chip from PEGDA hydrogel. They tested two different FDA-approved drugs, pitavastatin and irinotecan and achieved adjustable drug release. The advantage of this model was that results were obtained four days after drug administration [72].

3.3. Glioblastoma models in immunotherapy

Immunotherapy has been a major breakthrough in the treatment of various cancers in recent years, however not in glioblastoma. Glioblastoma is considered a "cold" tumor because it has low lymphocyte infiltration, sequestration in the bone marrow, and depletion of T lymphocytes [73]. There is also a highly immunosuppressive microenvironment characterized by downregulation of MHC I, overexpression of immunosuppressive cytokines, etc. But one of the major drawbacks in validating immunotherapeutic approaches is the lack of appropriate models. For models, it is critical to recapitulate the immune system, as the interactions between immune and cancer cells impact cancer development, progression, and treatment.

A suitable model for studying immunotherapy may contain autologous or allogeneic peripheral mononuclear cells. They should include T, B, and NK cells, but no granulocytes, erythrocytes, and platelets [73]. Models should also include tumor-infiltrating lymphocytes, as this allows for an intrinsic immune response and are also important for studying the tumor-specific efficacy of checkpoint inhibitors. There are two examples of organoid development for immunotherapy testing described below.

Neal et al. developed patient-derived organoids from more than 100 biopsies from various cancers. The tumors were mechanically processed and plated into collagen matrix into air-liquid interface. The organoids preserved TCR repertoire, tumor infiltrating cells,

Table 1
Clinical trials with the application of glioblastoma models.

Disease	Model	Application	ClinicalTrials.gov ID
Glioblastoma	Grafts of patient-derived glioblastoma stem cells onto autologous brain organoids	Glioma stem-like cells will be isolated from the tissue and IPSCs from fibroblasts or PBMCs. The organoids will be formed from PBMCs. The goal is to study invasion of glioma stem cells into brain organoids.	NCT05772741
Glioblastoma	Patient-derived organoid	Studying intra-organoid heterogeneity (genetic and epigenetic). Organoids that reflect temozolomide resistance and define oncogenic drivers in TMZ resistant cells. Set up organoids for drug testing.	NCT04868396
Glioblastoma	Patient-derived organoid	Patients will be treated based on their genetics and response of organoid to one of five selected drugs.	NCT05432518
Glioblastoma	Glioblastoma stem cell cultures and brain organoids	Organoids and glioblastoma stem cells will be developed from tissue and blood. Cilium-related transcriptome will be analysed. Glioblastoma stem cells in brain organoids will be treated with cilium-targeted drugs.	NCT05772767

Data was accessed on 26.6.2023 on ClinicalTrials.gov [76]. Search term for condition or disease was "glioblastoma" and for other terms was "3d model" and "organoid".

macrophages, Tc cells, Th cells, natural killer (NK), and natural killer T (NKT) cells and infiltrating $CD3^+$ T cells expressing the immune checkpoint surface receptor programmed cell death protein-1 (PD-1). At last, the organoids were treated with anti-PD-1 antibody nivolumab [74].

Jacob et al. developed patient-derived glioblastoma models. The tissue was mechanically dissected and place into ultralow attachment 6-well plates, which was placed on orbital shaker. The organoids retained genetic characteristics of original tumor as well as components of immune system. The organoids were then treated with the specific drug, based on signalling pathway based on somatic mutations. At last, the organoids were treated with CAR-T therapy [75].

4. Clinical trials including glioblastoma models and case studies

Glioblastoma models have high clinical potential, as reflected in recent clinical trials (Table 1). Two of the studies aim to develop organoids from blood cells and isolate glioblastoma stem cells from tissues. With the help of transcriptomics, they will analyse which pathways are related to glioblastoma stem cell invasion. One clinical trial will set up patient-derived organoids to study the mechanisms behind temozolomide resistance. At the fourth clinical trial patients will be treated with one of the five drugs based on organoid genetics and drug response.

The *in vitro* drug testing based on transcriptomics results has been successfully translated to the patient by Reed et al. [77]. Prior to sixth recurrence, the patient with Li-Fraumeni syndrome enrolled in clinical study. At sixth and seventh recurrence, spheroids were developed from tumor tissue and treated with panel of 12 drugs. Patient responded to the six drugs and osimertinib, an EGFR inhibitor, was chosen by the oncologist. After two months, recurrence occurred and again, spheroids were developed from tissue and tested for 12 drugs. Organoids were again responsive to osimertinib. The RNAseq was performed on all seven tumors and the last two tumors had elevated expression of *JAK1*, *STAT1*, *STAT2* and *mTOR*. Organoids from last 2 recurrences were treated with ruxolitinib (JAK1/2 inhibitor) and everolimus (mTOR inhibitor) and yielded optimistic results. The patient was then treated with the combination of both drugs and after more than 4 months had stable disease.

Similarly, Loong et al. prove the feasibility of using organoids in treatment prediction for glioblastoma patient [78]. They developed organoids from the glioblastoma tissue of 58-year old patient. The tissue was first digested with collagenase, seeded in Matrigel and cultured in neurobasal medium. Afterwards the genetic resemblance and genetic characteristics were determined by target sequencing. Based on the results, several drugs were selected which were then tested on organoids to predict drug sensitivity. Afterwards, the most efficient and suitable drug was chosen. The patient had encouraging partial response. The study shows that it is technically feasible to use organoids in drug selection.

5. Conclusion

Until now glioblastoma treatment has followed the standard path of surgery, radiation and chemotherapy. Unfortunately, so far, this has not led to significant improvement in patient life expectancy. Glioblastoma experimental models helped researchers understand the nature of the disease and provided clues for the design of new and specific therapies. Advanced 3D models, such as organoids, tissue-slice cultures, bioprinted models and organ on the chip present more suitable real tumor characteristics compared to standard 2D model, especially in terms of microenvironment generation. Nevertheless, each of this model has its own strengths and weaknesses, but in general formation of immune system, vasculature and recapitulation of intra- and intertumoral heterogeneity present the main challenge in model development. However, recent clinical trials and successful translation of in-vitro results from organoids to patient treatment prove the bright future of glioblastoma model application.

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Data availability statement

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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