

REVIEW

New hints towards a precision medicine strategy for *IDH* wild-type glioblastoma

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Glioblastoma represents the most common primary malignancy of the central nervous system in adults and remains a largely incurable disease. The elucidation of disease subtypes based on mutational profiling, gene expression and DNA methylation has so far failed to translate into improved clinical outcomes. However, new knowledge emerging from the subtyping effort in the *IDH*-wild-type setting may provide directions for future precision therapies. Here, we review recent learnings in the field, and further consider how tumour microenvironment differences across subtypes may reveal novel contexts of vulnerability. We discuss recent treatment approaches and ongoing trials in the *IDH*-wild-type glioblastoma setting, and propose an integrated discovery stratagem incorporating multi-omics, single-cell technologies and computational approaches.

Key words: *IDH*-wt glioblastoma, tumour microenvironment, multi-omics, precision therapy

INTRODUCTION

Glioblastoma (GBM) is the most common primary central nervous system (CNS) malignancy in adults with an annual incidence of 3 per 100 000.¹ It is a heterogeneous disease with a nearly universally fatal prognosis and, despite aggressive treatment with surgical resection and adjuvant chemo-radiotherapy, 85% of patients die within 2 years. Resistance to conventional therapies is related to several intrinsic properties of the tumour. For example, its diffuse infiltrative nature makes complete resection impossible, resulting in recurrence.² Moreover, the disease is further defined by microvascular proliferation, pseudopalisading necrosis and overt intratumoural heterogeneity.²

Historically, GBM diagnosis was based on histology, and classification limited to primary and secondary disease.³ However, the discovery of point mutations in genes

coding for the enzymes isocitrate dehydrogenase (*IDH*) 1 and 2 revolutionised the classification approach.⁴ *IDH* is a metabolic enzyme that catalyses the oxidation of isocitrate to alpha-ketoglutarate in the citric acid cycle⁵ (Figure 1). *IDH* mutation status was incorporated into the revised World Health Organization classification of brain tumours in 2016,⁶ thereby classifying GBM into two distinct entities: *IDH*-mutant (*IDH*-mt) GBM and wild-type (*IDH*-wt) GBM,⁶ although further molecular assessment suggests that *IDH*-mt GBMs align more closely with aggressive anaplastic astrocytomas.^{7–9} As such, the Consortium to Inform Molecular and Practical Approaches to CNS Tumour Taxonomy (cIMPACT-NOW) has proposed that the previously defined *IDH*-mt GBM is now referred to as astrocytoma, *IDH*-mt, grade IV. In addition, cIMPACT-NOW recommends the inclusion of *CDKN2A/B* homozygous deletion as a criterion for grade IV, *IDH*-mt astrocytomas.¹⁰ In this review, we will focus on *IDH*-wt GBM, which is often associated with single copy loss of chromosome 10 and gain in chromosome 7.¹¹ *IDH*-wt GBM manifests with significant interpatient differences and marked intratumoural heterogeneity. Additional frequent features include amplification of receptor tyrosine kinases such as epidermal growth factor receptor (*EGFR*) and platelet-derived growth factor receptor A (*PDGFRA*), mutations in telomerase reverse transcriptase (*TERT*)

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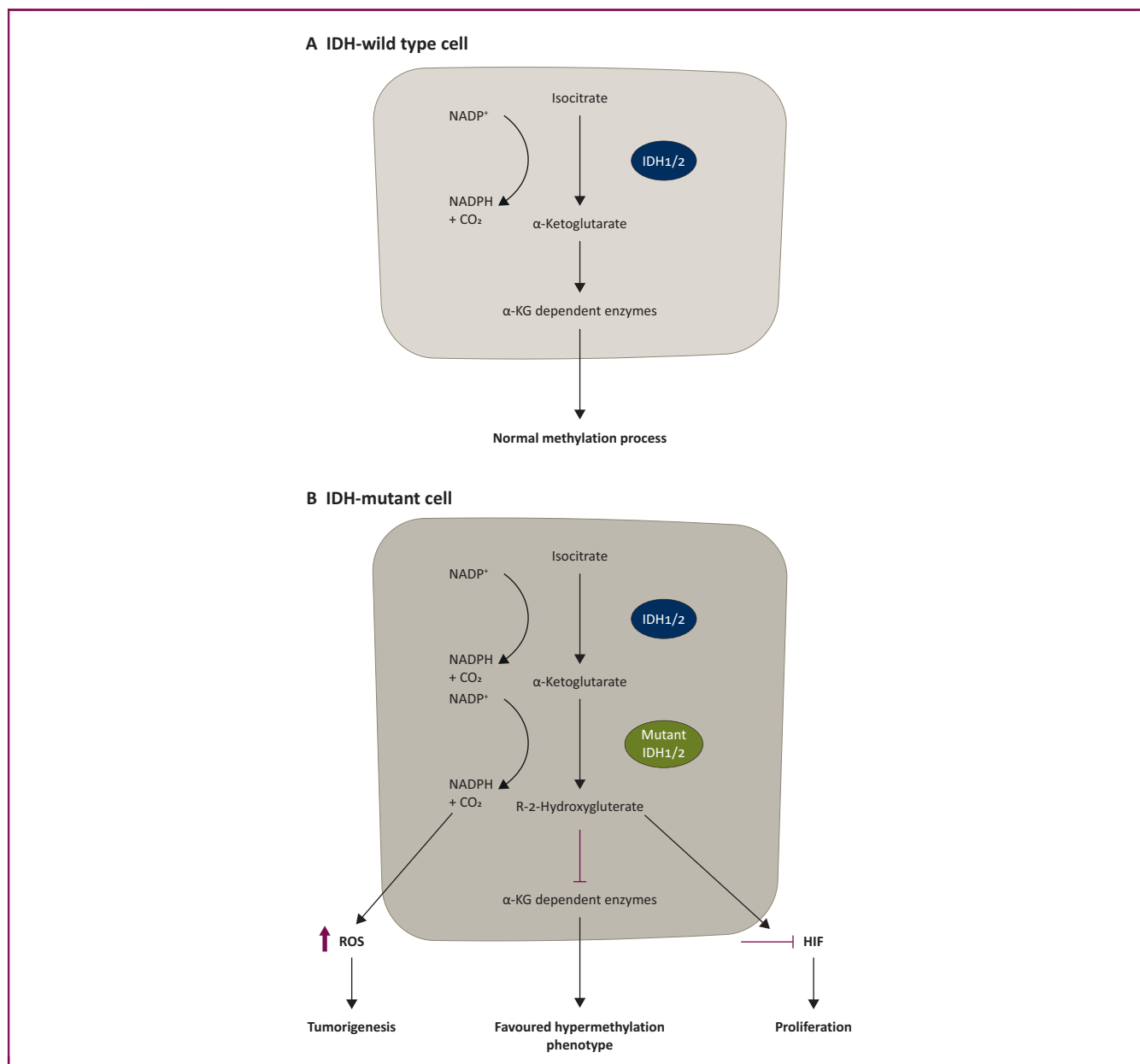


Figure 1. IDH signalling pathway in IDH-wild-type versus IDH-mutant cells.

Unlike aberrant IDH-mutant intracellular signalling, wild-type IDH expression elicits no major effects on cellular metabolism, production of ROS, tumorigenesis or proliferation. Cells expressing wild-type IDH favour a normal methylation pattern, compared with the favoured hypermethylation phenotype of IDH-mutant cells. HIF, hypoxia-inducible factor; KG, ketoglutarate; ROS, reactive oxygen species.

promotor and loss of the tumour suppressor gene phosphatase and tensin homolog (*PTEN*) (Figure 2). Interpatient differences are observed at the genome, transcriptome¹² and epigenetic level.⁴ Due to the heterogeneous nature of GBM, homogenous treatment approaches have to date led only to limited clinical advancement. It is thus clear that knowledge gained from diverse molecular profiling should direct future targeted therapeutic strategies. Here, we consider new knowledge emerging from GBM subtyping efforts, and reflect on how new learnings with respect to molecular subtyping and tumour microenvironment (TME)

characteristics may provide hints towards new precision targeting strategies.

MOLECULAR SUBTYPES

While classification based on IDH status supports the elucidation of distinct categories of malignant brain tumours, no novel therapeutic strategies have yet translated to clinical benefit based on IDH status. Hence, efforts to further stratify IDH-wt tumours are ongoing. Initial tumour stratification carried out before identification of IDH status

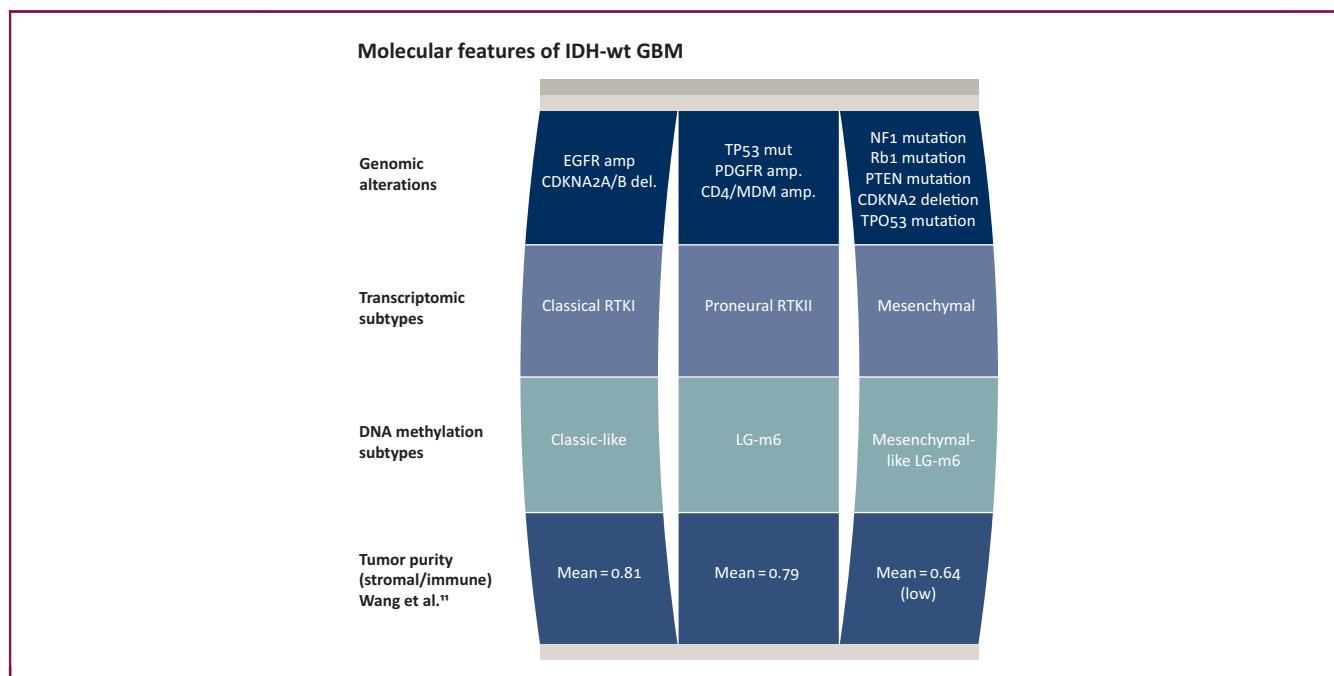


Figure 2. IDH-wild-type tumours are defined by distinct mutational and molecular features.

IDH-wt tumours harbour unique mutations which define molecular and transcriptomic subtypes, methylation subtypes, tumour purity.

amp., amplification; CDK4, cyclin-dependent kinase 4; CDKNA2, cyclin-dependent kinase inhibitor 2A; del., deletion; EGFR, epidermal growth factor receptor; GBM, glioblastoma; LG-m6, TCGA Pan-glioma (LGm) DNA methylation cluster 6; MDM, murine double minute; NF1, neurofibromatosis type 1; PDGFR, platelet-derived growth factor receptor; PTEN, phosphatase and tensin homolog; RB1, retinoblastoma 1; RTK, receptor tyrosine kinase; TP53, tumour protein p53; wt, wild-type.

by Phillips et al.¹³ in 2006 showed that molecular classes with enriched markers for proliferation, angiogenesis and the mesenchyme were predictive of overall survival (OS) and disease progression, with tumours commonly shifting towards the mesenchymal subclass upon recurrence. In 2010, Verhaak et al.¹⁴ identified four discrete transcriptomic subtypes of GBM: proneural, neural, mesenchymal and classical ($n = 202$ patients). Since then, the proneural phenotype was shown to correspond more closely to *IDH*-mt astrocytomas, younger age and secondary GBM. The initial favourable prognosis observed in the proneural subtype was due to the inclusion of secondary GBM.¹⁴⁻¹⁶ Classical GBMs display high-level *EGFR* amplification (97%) and few *TP53* mutations, whereas mesenchymal GBMs are underpinned by a high *NF1* mutational burden.¹⁴ Following emergence of the most recent classification of diffuse gliomas, and coupled with new data illustrating the influence of TME on GBM subtyping,¹⁷ Wang et al.¹² have now further refined GBM *IDH*-wt molecular subtypes. This subtyping approach is based on tumour-intrinsic transcriptomic signatures which are uniquely expressed by GBM tumour cells and not by tumour-associated host cells. In this context, three distinct subtypes have been shown to correlate with proneural, classical and mesenchymal tumours. The neural subtype was found to be largely comprised of samples with low tumour content and thus removed.¹⁸ Mutations in the *TERT* promoter have also been identified as a prognostic marker in this setting.¹⁹ Specifically, Killela et al.¹⁸ identified *TERT* promoter mutations (*TERT*-mt) in 83% of primary *IDH*-wt GBMs and have demonstrated that patients without the *TERT* mutation survive longer than *TERT*-mt patients (27 versus 14 months).

DNA methylation is a key factor in defining GBM heterogeneity. Patterns of DNA methylation in tumour cells play a significant role in defining the characteristics inherent to each GBM subgroup.^{15,20,21} *MGMT* promoter methylation is a well-known prognostic and predictive factor associated with response to alkylating agents such as temozolomide (TMZ).²² Indeed, integration of DNA methylation with RNA expression profiles in adult gliomas has revealed multiple novel glioma subgroups.⁴ Recent work has established two methylation clusters in *IDH*-wt; classic-like and mesenchymal-like (Figure 2). These clusters are associated with disease grade and patient prognosis and provide further insight into the impact of epigenetic alterations on glioma progression. de Souza et al.⁸ carried out a comprehensive DNA methylation longitudinal analysis of 200 gliomas from 77 patients. These analyses determined epigenetic patterns of malignant transformation from low to higher grade gliomas and identified epigenetic alterations from the *IDH*-mt cytosine-phosphate-guanine (CpG) island methylator phenotype (G-CIMP)-high subtype to the G-CIMP-low subtype which mimics *IDH*-wt primary GBM. These epigenetic alterations are predictive biomarkers for risk of malignant recurrence at early stage disease.⁸ Notably, *IDH*-wt epigenetic profiles did not significantly change upon recurrence. This study underscores epigenetic profiling as a robust classifier of GBM, which can identify key genetic alterations contributing to the aggressive *IDH*-wt phenotype. Additional studies to fully identify the evolutionary patterns driving these methylation changes are warranted.

Overall, molecular subtyping has made significant strides towards the elucidation of an improved understanding of

GBM heterogeneity. Nevertheless, these approaches have not yet generated clear insights into pathway dependencies which might be leveraged for the development of effective targeted therapies. Thus, a deeper characterisation of subtype specific tumour biology is needed.

TME CHARACTERISTICS

The GBM microenvironment consists of heterogeneous non-neoplastic cells, including glial cells, microglia, immune cells, vascular cells, reactive astrocytes and endothelial cells, in addition to various GBM cell subpopulations such as GBM stem cells (GSCs). These cell populations exist in several niches and have varying interactions with heterogeneous tumour cells.²³ GSCs are capable of remodelling the TME²⁴ and not only display different transcriptional and epigenetic heterogeneity depending on which niche they are derived from, but also interact between niches to leverage supportive cell signalling mechanisms.²³ Initial reports characterising GSCs suggested that this subpopulation could recreate heterogeneous tumours in a one-way hierarchical manner responsible for recurrence. However, it has recently become clear that GBMs are inherently plastic, and display stem cell properties to varying degrees.^{25,26} GBM cell populations therefore exhibit a dynamic heterogeneity and plasticity, with tumour equilibrium affected both by genetic background and microenvironmental cues such as oxygen concentrations or therapeutic pressure. Certainly, the role of tumour plasticity with respect to therapy resistance warrants significant attention.

Relative to other tumours, GBM presents an immunological 'cold' phenotype, defined by a low abundance of tumour-infiltrating lymphocytes (TILs).²⁷ Tumour-associated macrophages (TAMs) represent the most abundant component of the non-tumoural GBM TME, and, as part of the innate immune system, serve primarily to clear cellular debris via phagocytosis. TAMs, derived from microglia, resident brain macrophages and blood monocytes, are highly immunosuppressive and primarily involved in antigen presentation and cellular phagocytosis. It is noteworthy that in GBM the previous dual categorisation into M1-proinflammatory and M2-immunosuppressive macrophage phenotype has proven to be over-simplistic and does not provide a comprehensive representation of the complex activation states observed.²⁸ The degree to which macrophages infiltrate the tumour has been shown to correlate with a more aggressive clinical course and reduced OS.²⁹ Chen et al.³⁰ showed that macrophage-low patients ($n = 130$) display a greater OS compared with macrophage-high *IDH*-wt patients ($n = 201$). It was further shown that *PTEN* mutation culminates in increased TAM infiltration in the TME by up-regulation of the yes-associated protein 1 (*YAP1*) gene and lysis oxidase (*LOX*) expression in response to *PTEN* mutation. The resulting TAMs drive angiogenesis and glioma cell homeostasis via secretion of secreted phosphoprotein/osteopontin 1 (*SPP1*). The importance of TAM and *SPP1* in the TME was further demonstrated *in vivo* as *LOX* inhibition reduced tumour growth in a GBM orthoxenograft model.³⁰

TAMs were also associated with antiangiogenic therapy resistance.³¹ Interestingly, single-cell RNA sequencing (scRNA-Seq) analysis has also revealed increased expression of the macrophage recruitment factor gene *CSF1* in proneural tumours. Inhibition of the *CSF1* receptor (*CSFR1*), widely expressed in myeloid cells, has therefore been studied in transgenic models of proneural disease, and has been shown to improve survival outcomes in preclinical models.³² Unfortunately, despite the observed tumour regression in animals, *CSFR1* inhibitors failed to improve survival in patients, suggesting that TAMs acquire resistance to *CSFR1* inhibition.^{33,34} Nevertheless, efforts to reprogramme TAMs may prove important for eliciting response to immune therapeutics in a subset of GBM patients.²⁸ In particular, mesenchymal GBM has been shown to exhibit highest TAM infiltration,^{35,36} with significant macrophage content a histological signature of the subtype. Thus, notwithstanding the lack of overall clinical benefit observed to date, TAMs may yet represent a rational target in the mesenchymal setting.^{32,36}

Overall, the low abundance of TILs combined with the profoundly immunosuppressive TME in *IDH*-wt GBM provides major challenges for immunological treatments in this setting.³⁷ This aversive pro-neoplastic state is mediated through several mechanisms including overexpression of indoleamine 2,3-dioxygenase (*IDO*), production of interleukins and impaired antigen presentation.³⁸ Amankulor et al.³⁹ have identified differences in the infiltration of macrophages, microglia, monocytes and neutrophils between grade IV, *IDH*-mt astrocytomas and *IDH*-wt gliomas. *IDH*-wt GBM displays significantly higher CD45+ immune cell infiltration, including macrophages, dendritic cells, CD4 and CD8 T cells, microglia and B cells, than grade IV, *IDH*-mt astrocytomas^{39,40} (Figure 3). Polymorphonuclear cells (e.g. neutrophils) support extracellular matrix (ECM) remodelling allowing tumour progression and the establishment of new tumour vasculature.⁴¹ *IDH*-wt tumours (specifically within the mesenchymal subtype) have been shown to increase expression of immune checkpoint proteins such as programmed cell death ligand 1 (PD-L1).⁴² This prevents stimulation of effector T cells, which impairs the adaptive immune response. As these tumours exhibit a diverse immune cell infiltrate and harbour a TME that may be responsive to immunomodulating therapies, it is possible that *IDH*-wt mesenchymal tumours could be more responsive to combinatorial immune checkpoint inhibitor (ICI) treatment strategies.⁴³ For example, *IDH*-wt tumours display increased expression of PD-L1 and simultaneously display a dual up-regulation of *STAT3* and mammalian target of rapamycin (*mTOR*) pathways.⁴⁴ A combinatorial immune therapy protocol with *STAT3* or *mTOR* inhibition could potentiate the effects of ICIs.

Wang et al.¹² have also shown that *IDH*-wt GBM transcriptional subtypes display variations in the immune microenvironment. For example, the ESTIMATE computational tool, which infers stromal and immune cell presence from expression data,¹² reveals that the mesenchymal subtype has a significantly reduced tumour purity (Figure 2) compared with proneural and classical subtypes, with an

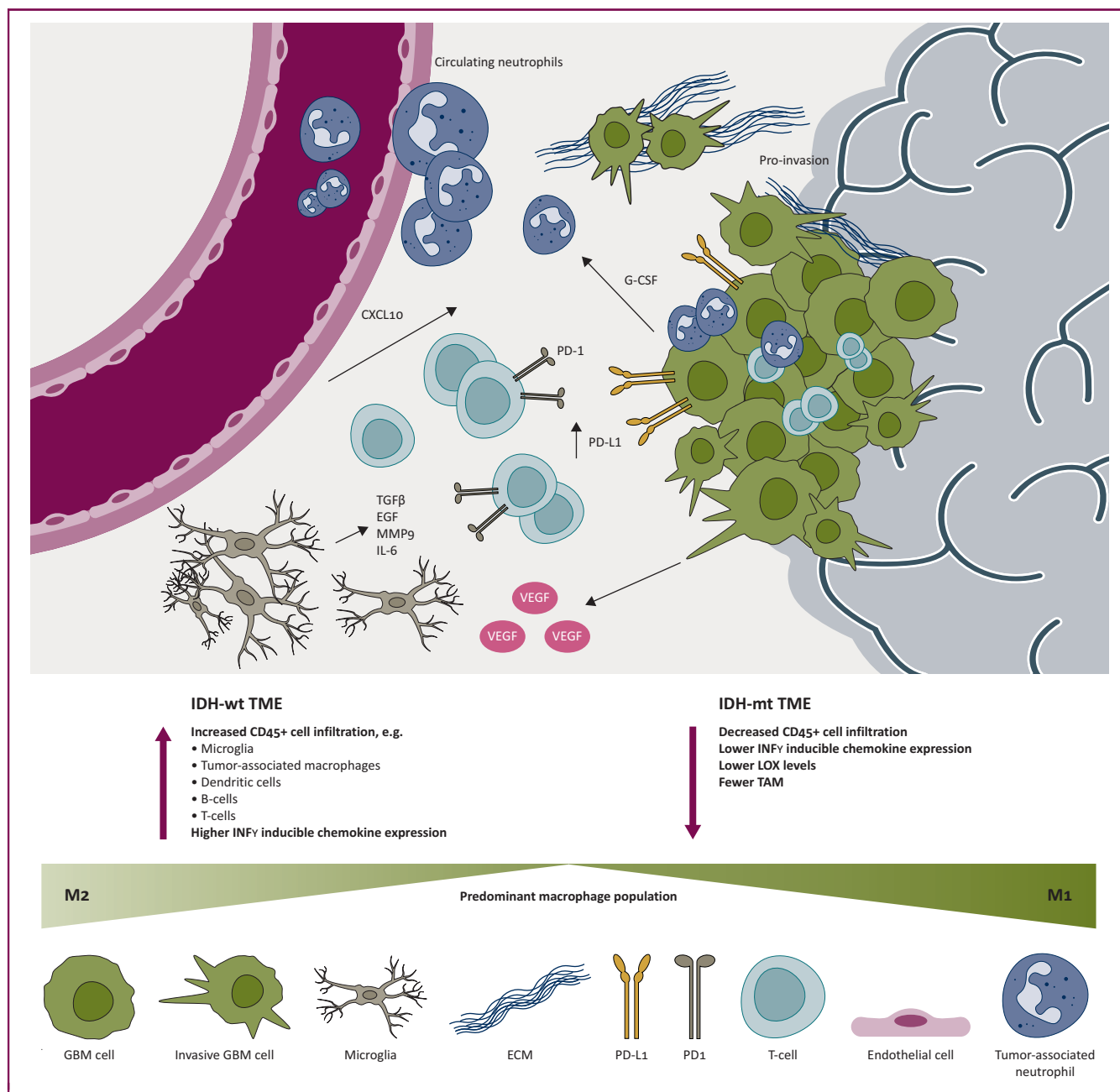


Figure 3. The *IDH*-wild-type GBM TME is highly heterogeneous, pro-invasive and immunosuppressive.

IDH-wt GBM display high levels of CD45+ cell infiltration including high concentrations of microglia and macrophages, B-cells and T-cells. *IDH*-wt tumours display greater VEGF, EGF, interferon (IFN)- γ -inducible chemokines (e.g. CXCL10), CCL2 concentrations, and greater proliferative and invasive capacity than *IDH*-mt tumours.

CCL2, C-C motif chemokine ligand 2; CXCL10, C-X-C motif chemokine ligand 10; ECM, extracellular matrix; EGF, endothelial growth factor; GBM, glioblastoma; G-CSF, granulocyte colony-stimulating factor; LOX, lysis oxidase; MMP, matrix metalloproteinases; mt, mutant; PD1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; TAM, tumour-associated macrophage; TGF, transforming growth factor; TME, tumour microenvironment; VEGF, vascular endothelial growth factor; wt, wild-type.

increased abundance of macrophages, microglia and neuroglia. Furthermore, the CIBERSORT⁴⁵ *in silico* cytometry method⁴⁶ further established an up-regulation of tumour-promoting, proinflammatory macrophage and neutrophil gene signatures, and significantly reduced levels of the natural killer cell gene signature in the mesenchymal subtype. These data suggest that *IDH*-wt GBM varies according to transcriptional context, and that the immune contexture

is partially dependent on *IDH* status. It therefore seems likely that *IDH*-wt tumours assigned to the mesenchymal subtype could respond better to immunotherapy due to its increased immune infiltrate, and might therefore be prioritised for future clinical trials with a targeted ICI.⁴⁷ In light of the subtype-specific differences in immune contexture discussed earlier, future trials may benefit further from a stratified, 'subtype-specific' design.

INTRATUMOURAL HETEROGENEITY AND PLASTICITY

The evolution of new and effective precision treatment strategies for *IDH*-wt GBM is hampered by considerable intertumoural and intratumoural disease heterogeneity. A study by Patel et al.⁴⁸ applied scRNA-Seq on five tumours and identified distinct heterogeneous intratumoural expression patterns between each GBM tumour. Moreover, this analysis first identified that tumours contain multiple and hybrid cell states according to classical, mesenchymal and proneural signatures. This study further showed that clinical outcome can be directly influenced by the proportion of each cellular subtype within a tumour, and specifically showed that greater intratumoural heterogeneity in the proneural subtype is associated with reduced survival.⁴⁸

GBM heterogeneity was further highlighted in a recent study by Neftel et al.²⁵ who identified four distinct and dynamic cellular states in *IDH*-wt tumours, modulated by both genetic drivers and the TME. It has been proposed that these states define the developmental potential of the tumour and intrinsic resistance to therapy. Specifically, combining scRNA-Seq data from 28 tumours with The Cancer Genome Atlas (TCGA) bulk data for 401 GBM specimens, revealed that malignant cells can exist in four reversible cellular states; neural-progenitor-like, oligodendrocyte-progenitor-like, astrocytic-like and mesenchymal-like.²⁵ These states may co-exist in individual tumours and the equilibrium between states is influenced by genetic alterations in *CDK4*, *PDGFRA*, *EGFR* and *NF1*, respectively. The cycling capacity between states and inherent plasticity further suggests that effective treatment hinges on targeting all four cellular states. Alternatively, cells might need to be propelled into a single targetable state *via* selective pressure on either the tumour or TME. Clearly, such intratumoural heterogeneity and dynamic cellular plasticity has significant implications on future therapeutic strategies in this intractable setting.

TREATMENT APPROACHES IN *IDH*-WT GBM

A comprehensive review focused on the current management of *IDH*-wt GBM with consideration also given to future directions has recently been published.⁴⁹

Kinase targeting

Kinase pathways represent an ostensibly valid therapeutic target in GBM. *EGFR* is overexpressed in 60% of *IDH*-wt GBMs,⁵⁰ which is often combined with expression of *EGFR* mutants or structural variants, whilst tumour suppressor phosphatase and the tensin homolog (*PTEN*) gene is mutated in 40% of cases.⁵¹ *EGFR* alterations include *EGFR* amplifications which often coincide with the oncogenic variant *EGFRvIII*. *EGFRvIII* functions to accelerate tumour growth and proliferation,⁵² whereas *PTEN*-related dysregulation of AKT/protein kinase B signalling cascade results in dysregulated cellular proliferation and aberrant *mTOR* activation.⁵³

Early evidence therefore suggested that targeting *PTEN*, *mTOR* and *EGFR* signalling cascades could hold promise;

however, this approach has thus far proved underwhelming.⁵⁴ These failures are exemplified when one considers the limited clinical effects observed with the tyrosine kinase inhibitors gefitinib, afatinib and lapatinib. Intervention with these agents, while preventing dimerisation of *EGFR* and thus inhibiting the receptor function, does not block the aberrant signalling downstream of the receptor and has yielded limited clinical benefit. For example, gefitinib did not improve patient OS in a phase II trial in recurrent GBM, or in a phase I/II trial in combination with radiation in newly diagnosed GBM. Similarly, despite being well tolerated in patients, afatinib and lapatinib have both largely failed in the clinic with minimal antitumour activity.⁵⁵⁻⁵⁷ Furthermore, challenges associated with small molecule targeting of *EGFR* were not overcome with *EGFRvIII* targeting peptide vaccines. ACT IV, a large phase III multicentre randomised, controlled trial (RCT) (NCT01480479) showed no survival benefit upon the addition of rindopepimut, an *EGFRvIII* peptide vaccine, to standard of care.^{58,59}

Resistance to *EGFR* therapy was, until recently, explained through positive signalling feedback, clonal evolution due to therapeutic pressure and limited delivery of larger molecules across the blood-brain barrier. More recently however, Nathanson et al.⁵² have shown that the rate at which cells recur with resistance following *EGFR* targeting therapy may not be a result of classical clonal evolution.⁵² Circular, extrachromosomal DNA (ecDNA) is employed by malignant cells to increase oncogene copy number without chromosomal amplification, and drives tumour resistance methods.⁶⁰ Indeed, it has been proposed that GBM tumours activate oncogenes through amplification of ecDNA, rather than classical chromosomal alterations.⁶¹ These oncogenic amplifications on ecDNA may also result in increased tumour heterogeneity and contribute to accelerated tumour evolution.⁵⁸ Indeed, Nathanson et al.⁵² show that resistance to *EGFR* tyrosine kinase inhibitors occur via elimination of mutant *EGFR* from circular, ecDNA. The presence of an *EGFRvIII* mutation on ecDNA results in an initial sensitivity to *EGFR* inhibition, however, upon withdrawal of therapeutic pressure, clonal *EGFRvIII* mutations rapidly re-emerge on this ecDNA, resulting in renewed resistance to therapy. This would suggest that oncogenic amplifications on ecDNA are essential in successful evasion of targeted therapies, resulting in significant drug resistance. Therefore, understanding the ecDNA mechanisms that drive this therapeutic resistance is needed in order to successfully target the oncogenic ecDNA amplifications in GBM. This highlights the diversity and complexity of mechanisms by which ecDNA promotes resistance in GBM.

Similarly, poor results and significant adverse effects have been seen with *mTOR* inhibitors across several trials.^{62,63} It is now well accepted that these failures result from inadequate inhibition of downstream signalling and positive feedback loops following single agent therapy. First generation *mTOR* inhibitors, including temsirolimus and everolimus, inhibit *mTOR* complex 1 (*mTORC1*) with little impact on *mTORC2* signalling. This may lead to compensatory

continued activation of AKT, secondary to continued *mTORC2*.⁶⁴ Next generation agents which target both *mTORC1* and *mTORC2* could circumvent the resistance observed when targeting *mTORC1* alone.⁶⁴ Nevertheless, trials which implement molecular discriminators to stratify discrete subgroups of patients may hold promise; for example, in a phase II RCT of newly diagnosed *IDH*-wt GBM patients (NCT01019434), standard of care treatment was compared with temsirolimus and radiotherapy.⁶⁵ While there was no significant improvement in OS and progression-free survival (PFS), hypothesis-generating subset analysis indicated that a small cohort of patients ($n = 13$) with phosphorylated-*mTOR*^{Ser2448} who received temsirolimus had a significantly increased OS when compared with patients negative for phosphorylated-*mTOR*^{Ser2448} (17.8 months versus 13.1 months; $P = 0.007$).⁶⁵

Overall, however, the reasons for failure of receptor tyrosine kinase-targeted therapy and other cytotoxic agents are multifaceted. Amid promising novel therapies, it would seem clear that monotherapy with targeted agents is unlikely to yield success in *IDH*-wt GBM patients. This is mainly due to diverse and adaptive intratumoural heterogeneity and ever-changing cellular states. Shrewdly chosen combinatorial regimens which exert synergistic effects may prove more successful.

Chimeric antigen receptor-T-cell therapy

Chimeric antigen receptor (CAR)-T-cell therapy has contributed greatly to the recent impetus in immunotherapy strategies in cancer.⁶⁶ Several impressive clinical trial results in CD19-positive acute lymphoblastic leukaemia and diffuse B-cell lymphoma led to Food and Drug Administration (FDA) approval of CAR-T-cell therapy in 2017.^{67,68} To date, results in the GBM setting have been variable; for example, Sampson et al.⁶⁹ observed that CAR-T-cell therapy in VM/Dk mice harbouring orthotopic SMA560vIII tumours resulted in a long-term cure which was maintained despite tumour rechallenge. Furthermore, O'Rourke et al.⁷⁰ provided evidence of CAR-T-cell-induced alteration of the TME where *EGFRvIII* expression levels decreased significantly in five of seven patients, and a robust cytolytic effect was induced at the disease site. Nevertheless, as evidenced in the ACT IV trial (NCT01480479), loss of *EGFRvIII* expression was observed in approximately 60% of patients irrespective of treatment⁵⁹ suggesting that decreased expression of *EGFRvIII* is a common and naturally occurring event. Moreover, post infusion specimens have revealed a compensatory increase in other immunosuppressive markers in the TME such as *IDO1*, transforming growth factor- β (*TGF β*), interleukin (*IL*)-10, *FOXP3* and *PD-L1*.⁷⁰ This limits the further expansion of implanted CAR-T cells, thus impairing a more prolonged clinical response.

Nevertheless, to address the specific challenge posed by the immunosuppressive landscape, a phase I study (NCT03726515) is currently investigating *EGFRvIII*-directed CAR-T-cell therapy in combination with pembrolizumab in

newly diagnosed *MGMT*-unmethylated GBM. In this context, it is hypothesised that targeting PD1 may reverse the immunosuppressive TME, causing increased CAR-T-cell efficacy.⁷¹ IL-13 receptor alpha 2 (*IL13R α 2*) represents another promising tumour-associated antigen target to reverse the immunosuppressive TME via CAR-T-cell technology.⁷² This high affinity IL13 receptor is significantly up-regulated in mesenchymal GBM compared with normal tissue.⁷¹ Initial studies have successfully delivered CAR-T cells targeting *IL13R α 2* intracranially. While an antitumour response has been elicited in a subset of patients, a survival benefit could not be established, given the limited number of patients ($n = 3$).⁷³ Most recently, a phase I clinical trial targeting *IL13R α 2* in combination with ICIs (ipilimumab and nivolumab) in GBM is being investigated (NCT04003649). A further phase I clinical trial studying the effects of CAR-T-cell therapy targeting *IL13R α 2* in recurrent/refractory malignant glioma (NCT02208362) has also been initiated. Data from these trials will provide important information on the safety, feasibility and optimal delivery approach for CAR-T cells and will assess the potential synergy between CAR-T-cell therapy and immune checkpoint blockade.

Overall, phase III CAR-T-cell data are urgently awaited. Furthermore, an improved understanding of GBM tumour heterogeneity and the underlying biology of the immunosuppressive TME, along with the identification of new antigen targets continues to be mandated.^{72,74}

Oncolytic virus therapy

Oncolytic virus (OV) therapy has emerged as a novel approach to circumvent the immunosuppressive TME. OVs based on adenovirus, herpes simplex virus, measles virus, reovirus, retrovirus, parvovirus, poliovirus and others have been assessed in GBM trials. The allure of OVs lies in their ability to selectively infect tumour cells having direct and indirect antineoplastic effects. OV-induced immunogenic cell death results in the direct release of pathogen- and damage-associated molecular patterns as well as pro-inflammatory cytokines, resulting in a massive recruitment and activation of immune cells. Tumour-associated antigens released from virally lysed cancer cells into the TME are cross-presented to T cells by antigen-presenting cells including dendritic cells and macrophages, or directly by the tumour cells, leading to the establishment of tumour-specific T-cell immunity. The adaptive immunity not only attacks the infected tumour cells, but also uninfected or distant disseminated tumour cells. Therefore, OVs have the potential to convert immunologically inert tumours into highly immune-reactive ones and induce potent, long-lasting antitumour immune responses.⁷⁵

Two OVs (DNX-2401 and PVS-RIPO) were recently granted a fast-track designation by the FDA for expedited drug review. DNX-2401 is an engineered tumour-selective adenovirus. A phase I clinical trial was conducted in 37 patients with recurrent malignant glioma and 20% of patients receiving a single intratumoural injection of DNX-2401 survived more than 3 years from treatment.

Analyses of post-treatment surgical specimens revealed direct virus-induced oncolysis and tumour infiltration by CD8+ and T-bet+ cells.⁷⁶ Cerebrospinal fluid samples from DNX-2401-treated GBM patients in another phase I trial revealed cytokine concentrations indicative of a pro-inflammatory microenvironment and a prolonged shift of the protumoural M2 macrophages toward pro-inflammatory M1 in post-treatment resection tissue.⁷⁷ The efficacy of poliovirus-derived PVS-RIPO is also being studied in GBM. Desjardins et al.⁷⁸ carried out a phase I clinical trial of recombinant poliovirus in 45 *IDH*-wt patients with recurrent GBM. Some 21% of patients treated with poliovirus OV were alive at 36 months in comparison with 4% survival at 36 months in the historical control group. This OV acts by selectively targeting the cell adhesion molecule CD155,⁷⁹ which is intimately involved in tumour immune escape strategies. In particular, CD155 blockade has been shown to enhance response to ICI.⁸⁰ Analysis of the TCGA and Repository for Molecular Brain Neoplasia Data (Rembrandt) databases revealed CD155 expression was highest in GBM compared with lower grade gliomas. This CD155 overexpression was most pronounced in mesenchymal and classical subtypes,⁷⁹ suggesting that patient stratification may further enhance treatment response rates for PVS-RIPO. Indeed, a study testing oncolytic measles virus in GBM xenografts identified constitutive interferon pathway activation as an efficacy determinant. Validation of this resistance profile in 10 GBM patients in a phase I trial revealed that virus replication in patient tumours was inversely correlated with expression of this resistance gene signature.⁸¹

A recent phase III RCT has further combined a retroviral and chemotherapeutic regime (Toca 511 and Toca FC) in recurrent anaplastic astrocytoma and GBM (NCT02414165). Toca 511 is a genetically modified retrovirus which encodes for the cytosine deaminase gene. Cytosine deaminase in turn converts the prodrug 5-fluorocytosine (Toca FC) to the cytotoxic compound 5-fluorouracil in cells infected with Toca 511.⁸² Patients underwent surgical resection and were randomised to either intracranial injection of Toca 511 followed by oral Toca FC or standard of care (lomustine, TMZ or bevacizumab) (NCT02414165). Unfortunately, the trial failed to meet its primary end point with no OS benefit evident in treatment arms (11.1 months versus 12.2 months; $P = 0.6154$).⁸³ Nevertheless, subgroup analysis indicated a survival benefit in second recurrence patients with *IDH*-mt and AA histology (Hazard Ratio = 0.102, $P = 0.009$). This survival benefit was not evident in the *IDH*-wt cohort. While further studies are needed, the potential of OV to reverse the GBM immunosuppressive microenvironment holds promise as an effective treatment, in particular when patient stratification and/or combinations with other immunotherapies can be implemented.

ICI therapy

Therapeutic targeting of immune checkpoint proteins via ICIs has been associated with significant clinical benefit in

several malignancies.⁸⁴ *PD-L1* has been shown to be highly expressed in *IDH*-wt GBM.^{85,86} Disappointingly, data from two recent phase III RCTs, CheckMate-143 (NCT02017717) and CheckMate-498 (NCT02617589), failed to show a survival benefit in both newly diagnosed and recurrent GBM patients treated with nivolumab.⁸⁷ Additionally, the more recent CheckMate-548 phase III RCT (NCT02667587), which evaluated the addition of nivolumab to standard of care in *MGMT* methylated newly-diagnosed GBM, has failed to meet its primary end point of PFS. OS data of this study are pending.⁸⁸

Despite disappointing outcomes from CheckMate-143 and CheckMate-498, recent work from Cloughesy et al.⁸⁹ and Schalper et al.⁹⁰ has yielded promise. A multicentre RCT studied the impact of neoadjuvant and adjuvant anti-*PD-1* blockade in recurrent GBM patients who were amenable to further surgical resection. Whilst patient numbers were small and therefore not sufficiently powered to assess survival impact, the neoadjuvant group demonstrated improved antineoplastic immune responses and OS rates (13.7 months versus 7.9 months). Further validation of these results is now needed. Overall, the advantage of commencing therapy in advance of surgery may lie in the greater antigen load before tumour debulking, thus fostering a stronger and more prolonged immunomodulatory impact.⁸⁹ Schalper et al. conducted a single-arm phase II clinical trial (NCT02550249) to assess the immune-biological effects of neoadjuvant and adjuvant anti-*PD1* blockade in 30 GBM patients.⁹⁰ Investigation into the changes in the immune microenvironment upon administration of neoadjuvant nivolumab revealed that nivolumab in a neoadjuvant setting promotes several anti-tumour immune effects, including increased immune cell infiltration, enhanced chemokine transcript expression and greater T-cell antigen receptor diversity among TILs.⁹⁰ Whilst ICI therapy has produced disappointing clinical results to date, it is possible that modifications to drug sequencing protocols may optimise clinical efficacy. Indeed, combination therapy with OV or neoadjuvant administration may allow priming of the immune system before ICI boosting. This approach is being taken in different cancer types⁹¹ including a GBM trial testing DNX-2401 with pembrolizumab (NCT02798406). ICI therapies may be further augmented by the combination of a *BRAF* and *MEK* inhibitor in a three-armed approach, as discussed by Killock⁹² in the melanoma setting. Likewise, priming *PD-1* and *PD-L1* with an *mTOR* or *STAT3* inhibitor may facilitate a more responsive environment for checkpoint inhibitors.⁹³ A recent review by Le Rhun et al. provides further discussion on molecular targeted therapy in GBM and discusses the necessity for redesigned clinical trials in this setting.⁹⁴

A high tumour mutational burden is observed in approximately 10% of recurrent GBM patients.⁹⁵ It was previously hypothesised that this hypermutant cohort may be more responsive to immune checkpoint blockade,⁹⁶ due to their neo-antigen load and antigen-targeting T cells.⁹⁷ Whilst *IDH*-wt GBM displays particularly low neo-antigen

concentrations contributing to immunotherapy resistance, it was thought that inducing the mutational state in a subset of GBM patients might elicit an immune response to checkpoint inhibition.⁹⁷ It was also hypothesised that TMZ could induce this hypermutant state upon recurrence, with TMZ-induced hypermutations most commonly associated with *MGMT* methylated gliomas with *IDH* mutations.⁹⁷ Notwithstanding these assumptions, it has yet remained unclear whether high mutational burden may support a superior immune response to immune checkpoint blockade. In an attempt to more accurately characterise the phenotypic and molecular features of hypermutated gliomas, Touat et al.⁹⁸ recently showed that a low *PD-1* blockade response rate was observed within a population of hypermutant gliomas that emerged following TMZ treatment. As such, it would seem that a TMZ-driven hypermutator phenotype does not guarantee an immune response to *PD-1* blockade, likely due to the concurrent presence of mismatch repair deficits and the subclonal nature of emergent neo-antigens. Indeed, pressure from alkylating agents alone is likely insufficient to induce a hypermutated phenotype which guarantees the response to immune checkpoint blockade observed in other malignancies.⁹⁸ Overall Touat et al.⁹⁸ have highlighted how disease-specific differences in the mutational landscape impact tumour response to immunotherapy.

Tumour treating fields

Tumour treating fields (TTF) has emerged as a novel therapeutic strategy in GBM, gaining recent FDA approval as an adjuvant therapy for newly diagnosed GBM patients following standard surgical resection and chemoradiation.⁹⁹ A transducer, worn by the patient, exerts both direct and indirect antineoplastic effects via continuous delivery of low intensity alternating electric fields (200 kHz). The 100-300 kHz range has been shown to selectively disrupt mitoses in rapidly dividing cells including the disruption of tubulin and septin complexes.^{100,101} The resulting impaired spindle function leads to aberrant chromatin segregation.¹⁰²

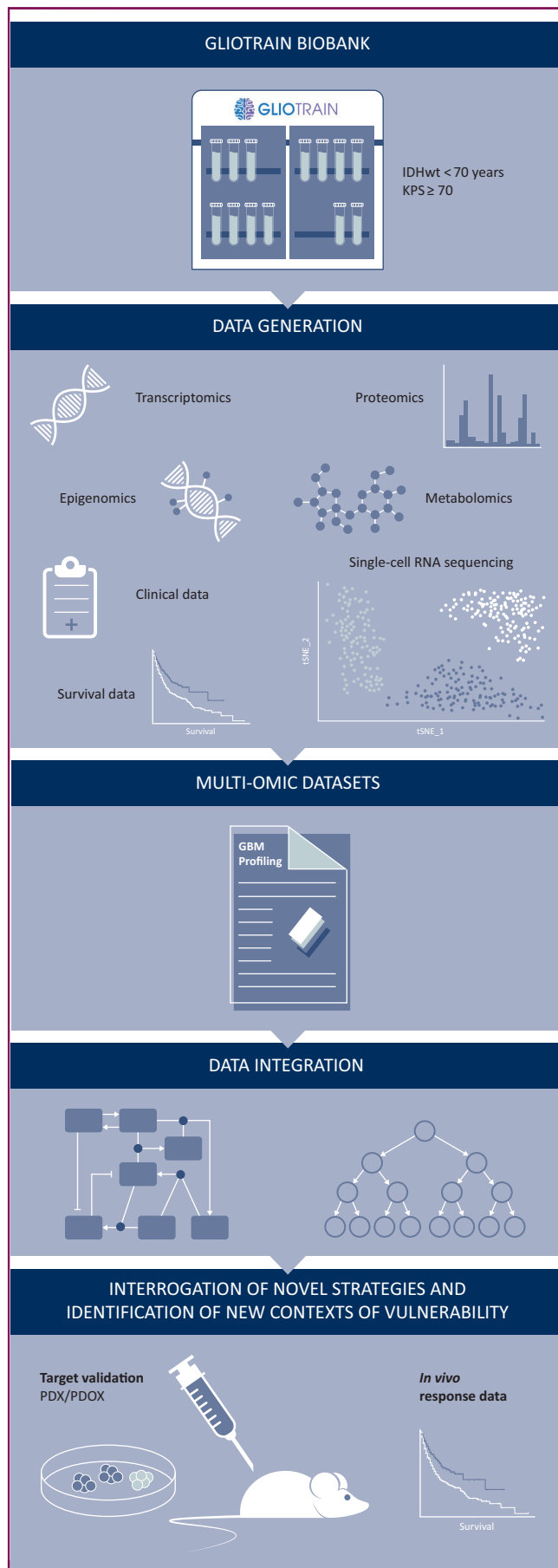
Overall, TTF has emerged as the only approach to elicit improved OS in *IDH*-wt GBM in recent years.¹⁰³ Stupp et al.¹⁰³ published data from a phase III RCT in 2017 which studied effects of TTF addition to patients undergoing standard chemoradiotherapy. When compared with standard of care alone, the addition of TTF improved both PFS (6.7 months versus 4 months) and OS (20.9 months versus 16 months). This patient cohort was largely comprised of *IDH*-wt GBM (92% TTF group versus 95% control group). Nevertheless, the wider implementation of TTF has several limitations. TTF cost remains a challenge with an average monthly treatment cost of €21,000.⁹⁹ Connock et al.¹⁰⁴ showed that combining TTF with TMZ in newly diagnosed GBM yielded a cost of approximately €500,000/year of life gained and would necessitate a cost reduction of 85% to become cost effective. In addition, users are required to wear the device for approximately 18 h per day. Stupp et al.¹⁰³ reported a compliance rate of 75%, although this

was in newly-diagnosed patients. Despite these limitations, optimisation of TTF in GBM treatment protocols remains an active area of research. Herrlinger et al. published data from a recent phase III RCT showing improved OS (48.1 months versus 31.4 months) in newly diagnosed *MGMT* hypermethylated GBM patients when treated with lomustine and TMZ in newly-diagnosed *MGMT* hypermethylated GBM.¹⁰⁵ The combined treatment of TTF and lomustine/TMZ has been shown to be safe and feasible in newly diagnosed GBM patients.¹⁰⁶ Two phase II clinical trials (NCT03405792, NCT03430791) aimed at studying the impact of TTF when delivered in combination with ICI in newly diagnosed and recurrent GBM (rGBM) are currently recruiting.

Neurotrophic tropomyosin receptor kinase fusions and BRAF alterations

Chromosomal rearrangements of neurotrophic tropomyosin receptor kinase (NTRK) genes occur in a significant number of GBM cases, leading to constitutively active chimeric receptors and oncogenic addiction. Gene fusions involving *NTRK1*, *NTRK2* or *NTRK3* (encoding TRKA, TRKB and TRKC, respectively) occur at varying frequencies in GBM, with *NTRK2* fusions the most commonly observed (up to 11% of GBM). *NTRK1* and *NTRK3* are observed in <1% of cases,^{107,108} These NTRK fusions drive ligand-independent activation of the TRK, resulting in activation of a variety of downstream cascades including RAS/RAF/MEK/ERK and PI3K/AKT pathways, ultimately promoting tumour cell proliferation and survival.¹⁰⁹ To date, it has been shown that overexpression of the neurofascin (NFASC)-*NTRK1* fusion gene in NIH 3T3 cells increases cell proliferation, colony formation and tumour formation in a xenograft model. Moreover, targeting *NTRK1* fusion transcripts with RNAi inhibits the proliferative phenotype of fusion gene-expressing cells.¹¹⁰ While this effect was not recapitulated with commercially available TRKA inhibitors (AZ-23, GW441756 or CEP-701), these data suggest that the presence of an NTRK fusion contributes to initiation or maintenance of selected GBM tumours and might represent a target of vulnerability in fusion-positive patients.¹¹⁰ Interestingly, larotrectinib, a selective pan-TRK inhibitor, has recently received FDA approval for use in cases of NTRK fusion-positive tumours. The inhibitor was tested in three basket trials (phase I, I/II and II; NCT02122913, NCT02637687 and NCT02576431, respectively) which included 14 patients harbouring primary CNS tumours. An overall response rate of 36% ($n = 5$) was demonstrated, including 14% complete responses ($n = 2$) and 21% partial responses ($n = 3$). While these data are encouraging, large-scale studies in the GBM setting are now warranted. Next generation therapeutics such as repotrectinib (ROS1, TRK and ALK inhibitor) are also currently under investigation in fusion-positive CNS malignancies (NCT04094610).¹¹¹

A subset of GBM tumours have also been shown to harbour mutations in v-Raf murine sarcoma viral oncogene homolog B (*BRAF*). In particular, this alteration is observed in the rarer *IDH*-wt epithelioid (eGBM) subtype, with



BRAFV600 mutations present in greater than 50% of these cases.¹¹² To date, targeting of mutant BRAF signalling has been studied in several trials, with both dabrafenib¹¹³ and vemurafenib (NCT01524978)¹¹⁴ showing promise in a small subset of BRAFV600 mutant tumours^{114,115}; The VE-BASKET study of BRAFV600 mutant, nonmelanoma cancers assessed the effect of vemurafenib in $n = 24$ patients with gliomas. Vemurafenib treatment resulted in a durable antitumour response in a cohort of *IDH1/2* wt low-grade gliomas, with the greatest effect seen in pleomorphic xanthoastrocytoma patients ($n = 7$). This positive antitumour response was not reflected in the higher-grade gliomas ($n = 11$) where only one partial response and five cases of stable disease were observed. Indeed, two cases of stable disease greater than 6 months were recorded, but no patient showed a complete response. While these data suggest that vemurafenib has utility in BRAFV600 mutant gliomas, responses observed within the trial were variable and dependent on histological subtype. Moreover, patients lacked additional genomic characterisation which would be required to further interrogate observed treatment response patterns. Nevertheless, BRAF may be a targetable oncogene in a small subgroup of *IDH*-wt GBM patients. Further validation of this approach is outstanding.¹¹⁴

FUTURE DIRECTIONS: IDENTIFYING NEW *IDH*-WT SPECIFIC CONTEXTS OF VULNERABILITY

To date, several efforts have been made to study the molecular underpinnings of GBM using high-throughput single 'omic profiling (whole genome sequencing, methylomics, RNA sequencing, microarray methods, reverse phase protein array, mass spectrometry and deep metabolomics)^{12,14,116} with an aim to identify altered genetic and epigenetic tumour landscapes, explore the differential expression of mRNA and protein and identify new contexts of vulnerability. However, a complete and systematic understanding of the complexities of disease heterogeneity requires the generation and integration of multiple molecular profiles (multi-omics). These profiles may subsequently be interrogated using advanced network analyses that include specific signalling pathways (Figure 4). Such machine network topology information, analysis of master regulators or mechanistic and stochastic modelling of learning approaches serve two purposes: (i) classification and integration of large amounts of diverse data sets; and (ii) mechanistic analysis using 'fine grained' models that

Figure 4. Proposed integrative systems medicine framework for precision treatment in *IDH*-wild-type GBM.

Omic data are collected from genomic, proteomic, metabolomic, epigenomic, immunomic, transcriptomic and single-cell analyses. Machine learning and artificial intelligence (AI) methods support the clustering, classification and integration of 'omics and clinical data resulting in the generation of prediction profiles and novel contexts of vulnerability. Such a novel systems biomedicine framework could identify new actionable pathways, biomarkers and therapeutic targets in *IDH*-wt GBM. These therapeutic targets and novel combinatorial approaches will be interrogated in state of the art patient derived organoid (PDO) and patient derived xenograft (PDX) models.

GBM, glioblastoma; KPS, Karnofsky performance score; tSNE, t-distributed stochastic neighbor embedding; wt, wild-type.

simulate biochemical pathways and allow prediction of new drug targets, combinations and personalised treatments. As discussed, novel multi-omic studies at the single-cell level are also now emerging which allow for the simultaneous integration of bulk gene expression, epigenomic, proteomic and metabolomic data thus providing deeper insight into the cellular diversity and genetic heterogeneity present within the TME.²⁵ In short, a major goal of integrative ‘multi-omics’ is to identify combined variables or biomarkers from multi-omics data that can predict phenotypic outcomes such as therapeutic responses and prognosis in cancer patients associated with their *IDH* status. This approach requires access to large, well-curated datasets such as that generated by Brennan et al. (500 GBM tumours),¹¹⁷ the Rembrandt database (671 patients),¹¹⁸ Ivy Glioblastoma Atlas Project (Ivy GAP) cohort (41 patients)¹¹⁹ or the Glioma Longitudinal Analysis (GLASS) consortium (257 patients).¹²⁰ Access to these datasets is expected to unravel the complex interactions between the genome, transcriptome, epigenome, metabolome and significantly improve the understanding of GBM hierarchies.

In order to better exploit molecular subtypes for therapy selection and optimise the use of existing drugs, we should also exit the paradigm of ‘one marker fits one targeted drug regimen’. We are actively working to refine the molecular stratification of *IDH*-wt GBM from a functional perspective, applying a systems approach for identifying targetable contexts of vulnerabilities and biomarkers that will be validated in state-of-the-art preclinical models. In a fully integrated approach, the EC funded cross-sectoral European training network ‘GLIOTRAIN’ (www.gliotrain.eu) is currently leveraging genomic, transcriptomics (bulk and single-cell), epigenomic and proteomic data (underpinned by a novel computational modelling framework) to interrogate TME, metabolic and immunological features of *IDH*-wt tumours (Figure 4). The overall objective of GLIOTRAIN is to identify and interrogate novel therapeutic strategies for application in *IDH*-wt GBM while simultaneously unravelling disease resistance mechanisms.

Conclusion

Targeting *IDH*-wt GBM remains one of the most difficult challenges in oncology today due to several obstacles, including the pervasiveness of signal transduction feedback loops and pathway redundancy, effects of tumour heterogeneity on the positive selection of drug-resistant subclones and an immunosuppressive TME. Elucidation of *IDH*-dependent functional relationships, genetic interactions and unique signalling dependencies are required to identify more effective therapeutic strategies. Success will leverage new knowledge gained from integrated bulk and single-cell multi-omic studies which have already assigned GBM into potentially targetable subtypes.^{12,14} Each *IDH*-wt subtype may ultimately be defined by differing vulnerabilities which could be targeted in the future according to the paradigm of precision medicine.

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