"Zooming in" on Glioblastoma: Understanding Tumor Heterogeneity and its Clinical Implications in the Era of Single-Cell Ribonucleic Acid Sequencing

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Received, November 19, 2019.
Accepted, April 30, 2020.
Published Online, July 16, 2020.

Glioblastoma (GBM) is the most common primary brain malignancy in adults and one of the most aggressive of all human cancers. It is highly recurrent and treatment-resistant, in large part due to its infiltrative nature and inter- and intratumoral heterogeneity. This heterogeneity entails varying genomic landscapes and cell types within and between tumors and the tumor microenvironment (TME). In GBM, heterogeneity is a driver of treatment resistance, recurrence, and poor prognosis, representing a substantial impediment to personalized medicine. Over the last decade, sequencing technologies have facilitated deeper understanding of GBM heterogeneity by "zooming in" progressively further on tumor genomics and transcriptomics. Initial efforts employed bulk ribonucleic acid (RNA) sequencing, which examines composite gene expression of whole tumor specimens. While groundbreaking at the time, this bulk RNAseq masks the crucial contributions of distinct tumor subpopulations to overall gene expression. This work progressed to the use of bulk RNA sequencing in anatomically and spatially distinct tumor subsections, which demonstrated previously underappreciated genomic complexity of GBM. A revolutionary next step forward has been the advent of single-cell RNA sequencing (scRNAseq), which examines gene expression at the single-cell level. scRNAseq has enabled us to understand GBM heterogeneity in unprecedented detail. We review seminal studies in our progression of understanding GBM heterogeneity, with a focus on scRNAseq and the insights that it has provided into understanding the GBM tumor mass, peritumoral space, and TME. We highlight preclinical and clinical implications of this work and consider its potential to impact neuro-oncology and to improve patient outcomes via personalized medicine.

KEY WORDS: Glioblastoma, RNA sequencing, Single-cell RNA sequencing

Neurosurgery 88:477–486, 2021 DOI:10.1093/neuros/nyaa305 www.neurosurgery-online.com

Glioblastoma (GBM) is the most common primary brain malignancy in adults and is almost uniformly lethal.[1,2] Despite extensive efforts to improve patient outcomes, median survival remains less than 20 mo, and less than 5% of patients survive beyond 5 yr.[3-5] GBM is particularly notorious for its intratumoral heterogeneity and infiltrative nature, both of which drive treatment resistance, recurrence, and poor prognosis.[6,7] Over the last decade, technological advances have allowed us to better understand this heterogeneity with progressively higher resolution, from examining gene expression in whole tumors to spatially distinct subsections of tumors and now to single cells comprising tumors and the tumor microenvironment (TME) (Figure 1). We review this progression in our understanding of GBM heterogeneity, with particular attention to seminal studies employing single-cell ribonucleic acid (RNA)seq (scRNAseq). We present representative studies driving the paradigm shift in our understanding of GBM and its TME at

ABBREVIATIONS: MGMT, 06-methylguanine-DNA methyltransferase; **N2M2,** NCT Neuro Master Match; **CNS,** central nervous system; **GBM,** Glioblastoma; **IDH,** isocitrate dehydrogenase; **LITT,** laser interstitial thermal therapy; **RNS,** ribonucleic acid; **scRNAseq,** single-cell RNA sequencing; **TAM,** tumor-associated macrophage; **TME,** tumor microenvironment

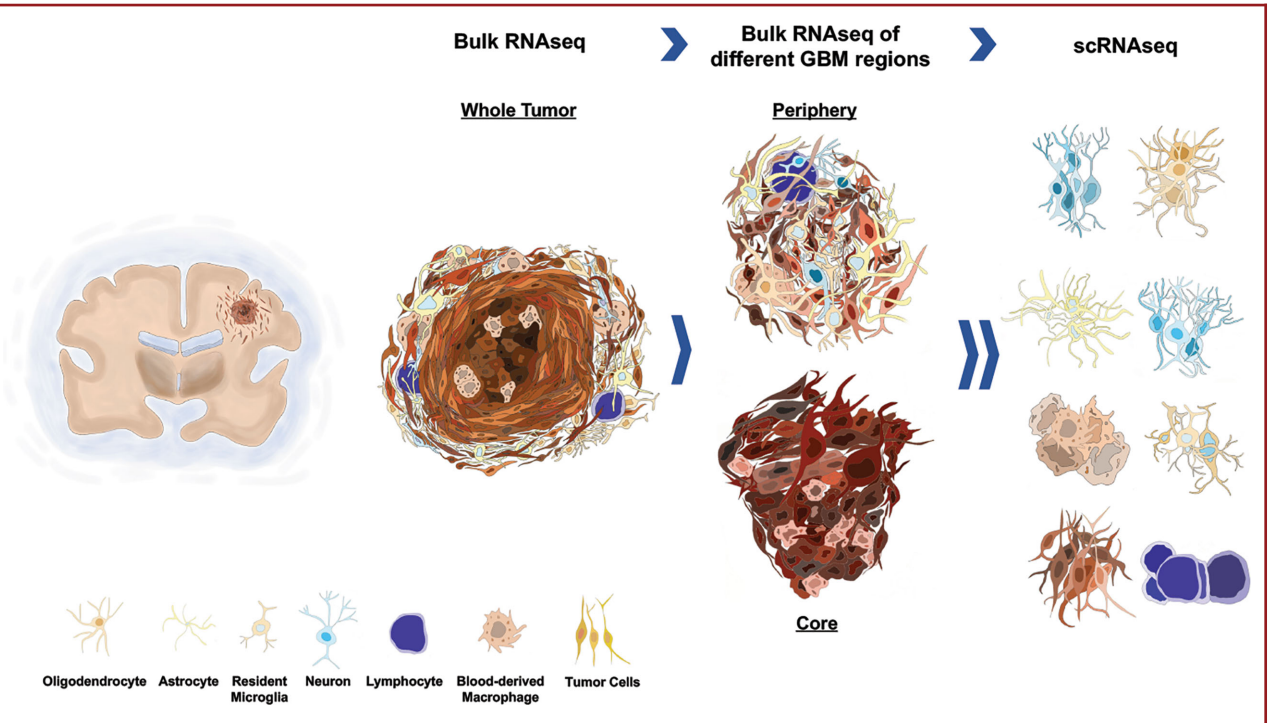

FIGURE 1. *Graphical depiction of progressively increased resolution achieved and information obtained through advances in different sequencing methods over time. Left panel, GBM tumor mass. Coronal section demonstrating presence of parietal GBM tumor mass with infiltration into surrounding tissue. In the next panel is shown the traditional method of bulk RNAseq of entire tumor specimens. This method provides limited resolution of RNA expression and masks underlying intratumor heterogeneity. Next panel, "Zooming In" Part 1: Bulk RNAseq of different anatomic regions of GBM. Distinguishing between anatomically distinct subsections of GBM tumors provides additional insight into the heterogeneity of cell populations and gene expression from different areas of the same tumor (ie, tumor core vs tumor interface/periphery). Right panel, "Zooming In" Part 2: scRNAseq of tumor mass/periphery. Single-cell RNAseq allows for determination of gene expression at the level of single cells from different areas within the same tumor. This technique unmasks otherwise unrecognized heterogeneity within tumors, providing deeper insight into the genomic landscape of GBM.*

the single-cell level (Table). scRNAseq may soon be regularly incorporated into existing clinical workflows, and it is poised to impact the neuro-oncology ecosystem surrounding GBM. This review provides a primer for neurosurgeons, neuro-oncologists, and radiation oncologists on historical advances and emerging directions of scRNAseq in GBM, such that they may prepare for and fully harness the potential of this exciting new technology.

HISTORICAL SEQUENCING APPROACHES TO GBM

Background

An important foundation for scRNAseq efforts in GBM was established by The Cancer Genome Atlas project in 2008. This National Institutes of Health-led work provided detailed genomic analysis of 206 GBMs using microarrays and high-throughput sequencing.[8] Their investigation yielded novel observations into the roles of *ERBB2*, *NF1*, *TP53*, and *PIK3R1* in the pathogenesis of GBM.[8] Additionally, their integration of clinical treatment data with genomic analyses revealed the link between 06-methylguanine-DNA methyltransferase (MGMT) promoter methylation and a hypermutator phenotype in treated GBM.[8] The findings also supported the notion that patients with activating mutations in *PIK3R1* could benefit from a PI(3)K or PDK1 inhibitors. Verhaak et al[9] expanded upon these efforts in 2010 to define four subtypes of GBM based on gene expression signatures: classical, mesenchymal, proneural, and neural. These were noted to be driven primarily by signaling via *EGFR*, *NF1*, *PDGFRA/IDH1*, and neuronal markers, respectively.[9] The most consistent clinical association with tumor subtype was age; younger patients were overrepresented in the proneural subtype, which trended toward a longer survival. This classification scheme represented a substantial step forward in understanding the heterogeneous nature of GBM, in which different subtypes had distinct gene signatures, druggable targets, responses to therapy, and putative lineages.[9] Their work illustrated how aggressive treatment significantly reduced mortality in classical (HR = 0.45; $P = .02$) and mesenchymal (HR = 0.54; $P = .02$) subtypes, while it did not alter survival in the proneural subtype (HR = 0.8;

TABLE. Studies Included in Literature Review

Authors and year	Sample characteristics	Key methods	New public datasets	Highlights
Bulk RNAseq studies of whole gbm tumor specimens				
The Cancer Genome Atlas, 2008[8]	206 GBMs	• Whole-genome sequencing (Sanger method) • DNA copy number analysis • Gene expression analysis • DNA methylation analysis	TCGA dataset	• Foundational work that established the utility of and infrastructure for integrated, large scale genomic analysis and public data sharing • Provided network view of pathways altered in GBM development • Demonstrated link between MGMT promoter methylation and hypermutator phenotype • Identified deregulation of RB, p53, and RTK/RAS/PI3K pathways as obligatory events in most GBMs • Suggested opportunities for targeted therapies based on particular patient mutations rather than a "one size fits all" approach • Suggested potential mechanisms of GBM treatment resistance
Verhaak et al, 2010[9]	200 GBMs and normal samples	• Gene expression analysis via multiple microarray platforms	TCGA dataset	• Established the commonly used Verhaak classification of four GBM subtypes based on distinct gene expression: classical, mesenchymal, proneural, and neural driven primarily by signaling via EGFR, NF1, PDGFRA/IDH1, and neuron markers, respectively • Demonstrated heterogeneous treatment effects and clinical outcomes for each subtype
Bulk RNAseq of different anatomic regions of GBM				
Gill et al, 2014[12]	69 GBMs	• Image-guided biopsies from contrast-enhancing tumor core and non-contrast-enhancing tumor periphery • Bulk RNAseq	Gene expression omnibus GSE59612	• Applied RNAseq to the tumor periphery, whereas previous work had primarily examined the tumor core • Demonstrated that molecular signature and histopathologic features varied greatly across regions within a single tumor • Identified common patterns that relate gene expression in the tumor core to that of the tumor periphery
Puchalski et al, 2018[13]	41 GBMs	• Laser microdissection used to isolate RNA from anatomically distinct regions of GBM: leading-edge, cellular tumor, pseudo-palisading cells around necrosis (PAN), microvascular proliferation (MVP)	Ivy GBM atlas http://glioblastoma.alleninstitute.org/	• Demonstrated distinct gene expression in different histologically-defined anatomical regions of GBM • Mapped molecular information to histologic hallmarks of GBM • Gene ontology terms for each area: • Leading-edge: neuronal systems • Cellular tumor: glial cell differentiation • PAN: stress, hypoxia, immune responses • MVP: angiogenesis, immune regulation, response to wounding
scRNAseq of GBM				
Patel et al, 2014[18]	5 GBMs (430 cells)	• scRNAseq	Gene expression omnibus GSE57872	• Discovered that individual GBMs could contain subpopulations of cells from distinct Verhaak subtypes • Identified stem-like compartment distinct from differentiated cells which was treatment-resistant and paralleled progenitor cells in developing brain • Noted four distinct gene expression meta-signatures related to hypoxia, complement/immune response, oligodendrocytes, and cell cycle • Correlated increased tumor heterogeneity with a dose-dependent decrease in overall survival

TABLE. continued

Authors and year	Sample characteristics	Key methods	New public datasets	Highlights
Darmanis et al, 2017[7]	4 GBMs (3589 cells)	• scRNAseq of GBM tumor core and peritumoral region	http://www.gbmseq.org/	• Identified and characterized the infiltrating tumor cells at the migrating front of GBM • Found a consistent gene expression signature of infiltrating cells across samples, suggesting a common mechanism of infiltration • Specific upregulation of genes involved in size regulation, energy production, inhibition of apoptosis, regulation of cell-cell adhesion, and CNS development • Noted that cells in the peritumoral region resembled their respective tumor cores
Venteicher et al, 2017[19]	16 IDH-A and IDH-O gliomas	• scRNAseq	Gene expression omnibus GSE89567	• Discovered that the two classes of IDH-mutant gliomas were distinguished by particular genetic events and differently composed TMEs rather than by distinct progenitor cells and lineages • Noted that increased glioma grade was associated with enhanced proliferation of malignant cells and a larger number of undifferentiated cells
scRNAseq of GBM TME				
Müller et al, 2017[25]	11 GBMs	• Isolated TAMs from patient samples scRNAseq of GBM TME	European genome-phenome archive repository EGAS00001002185 EGAS00001001900	• Identified distinct phenotypes and gene signatures in TAMs from peripheral blood vs brain-resident microglia • Blood-derived TAMs upregulated immunosuppressive (M2 phenotype) cytokines • Brain-resident microglia expressed more pro-inflammatory (M1 phenotype) features • Found spatially distinct locations of different GBM TAMs • Blood-derived TAMs in necrotic tumor core and perivascular regions • Brain-resident microglia in tumor leading edge • Correlated increased expression of blood-derived TAMs, but not brain-resident microglia, with inferior patient survival
Darmanis et al, 2017[7]	See above			• Found that tumor core was preferentially enriched in macrophages expressing anti-inflammatory and pro-angiogenic factors • Noted that peritumoral spaces were preferentially enriched in brain-resident microglia expressing pro-inflammatory markers
Venteicher et al, 2017[19]	See above			• Discovered that increased GBM tumor grade is associated with an increase in macrophage (vs microglia) expression programs in the TME

$P = .4$).[9] This comprehensive work and genomic classification laid the groundwork for an improved molecular understanding of GBM.

Limitations of Bulk RNAseq

While revolutionary at the time, these studies were limited by the use of bulk RNAseq, which provides an average of expression profiles for tumor specimens as a whole. This method lacks the resolution to distinguish between contributions from diverse subpopulations within the same specimen, which we increasingly understand to be drivers of treatment resistance and poor prognosis. Bulk RNAseq may therefore underestimate tumor genomics and complicate the development of personalized therapies and reliable biomarkers.[10]

"ZOOMING IN" PART 1: BULK RNASEQ OF DIFFERENT ANATOMIC REGIONS OF GBM

Recognizing this limitation, Sottoriva et al[11] sought to better understand these GBM subpopulations in their 2013 study by examining intratumoral heterogeneity from different anatomic regions within 11 GBM samples. During tumor resection, the authors used fluorescence-guided sampling to obtain 4 to 6 spatially distinct GBM fragments at least 10 mm^3 apart. Superficial fragments were taken during early tumor debulking, and progressively deeper fragments were taken as the operation progressed. After analyzing copy number variation, gene expression (via bulk RNAseq), and single-molecule mitotic levels from these distinct tumor fragments, they found that multiple GBM molecular subtypes were frequently present within a single tumor.[11] Of the 10 patient tumors analyzed, 2 tumors contained 3 Verhaak subtypes (classical/proneural/neural and classical/mesenchymal/neural, respectively), and 4 tumors contained 2 Verhaak subtypes (classical/neural, mesenchymal/proneural, mesenchymal/classical, and mesenchymal/proneural, respectively). The remaining 4 tumors contained 1 Verhaak subtype each (classical, mesenchymal, mesenchymal, and proneural, respectively).[11] These findings shed new light on intratumoral heterogeneity and offered early insights into the clonal evolution and drivers of treatment resistance in GBM. These findings also suggested that a single biopsy is insufficient to characterize each GBM and that such biopsies could significantly underestimate the composite landscape of relevant tumor mutations.[11]

In their 2014 study, Gill et al[12] performed image-guided biopsies and bulk RNAseq from 2 regions of GBM in 69 patients. They distinguished between the tumor core, a highly cellular contrast-enhancing region with substantial angiogenesis and blood-brain barrier breakdown, and the tumor periphery, a noncontrast-enhancing edematous brain region containing infiltrating tumor cells.[12] This distinction is clinically relevant, as the cells on the periphery are often left behind following surgical resection and may subsequently promote tumor recurrence. The authors[12] found that samples from the tumor core largely resembled the proneural, classical, or mesenchymal classes of GBM and contained substantial cellular density, necrosis, microvascular proliferation, and mitoses. Meanwhile, the nonenhancing tumor margins largely resembled the neural GBM subtype and contained a combination of glial cells and non-neoplastic cells such as neurons, oligodendrocytes, reactive astrocytes, and microglia.[12] Analyses of composite gene expression from the tumor margins should, therefore, consider previously underappreciated contributions from these non-neoplastic brain cells.[12] The authors[12] also identified conserved patterns relating gene expression of the tumor core to that of the tumor margins in a Verhaak subtype-specific pattern; for example, the margins of proneural tumors tended to have increased gene expression largely distributed to oligodendrocyte progenitor-like cells, whereas increased gene expression in the margins of mesenchymal tumors largely distributed to astrocytes and microglia. Due to these specific relationships, the characteristics of the postsurgical residual tumor margins could potentially be inferred from the characteristics of the tumor core deduced after resection.

An important next step was taken by Puchalski et al in their 2018 study. They used laser microdissection to isolate RNA from anatomically distinct regions of 41 GBMs. As defined by the authors, these regions included the leading edge (outermost boundary of the tumor), infiltrating tumor (intermediate zone between leading edge and cellular tumor), cellular tumor (tumor core), pseudo-palisading cells around necrosis (cells arranged around the perimeter of the necrotic tumor core), and microvascular proliferation (2 or more blood vessels sharing a common vessel wall and arranged in distinct shapes with interconnected blood vessels).[13] Using bulk RNAseq, they found that samples from these different anatomical regions were largely distinct in gene expression. Their analysis showed gene ontology terms for these regions were as follows: leading-edge—neuronal systems (cognition, dendrite development, and neurogenesis); cellular tumor—glial cell differentiation; pseudo-palisading cells around necrosis—stress, hypoxia, immune responses; microvascular proliferation—angiogenesis, immune regulation, response to wounding.[13] This work, publicly available as the Ivy GBM Atlas, demonstrated that GBM was even more heterogeneous than previously suspected.[13] There was marked heterogeneity between different regions within individual tumors and even greater heterogeneity than previously expected between similar anatomic areas of different patients' tumors.[13]

"ZOOMING IN" PART 2: SINGLE-CELL RNASEQ OF GBM—BACKGROUND

scRNAseq Overview, Current Status, and Limitations

The next step forward, following bulk RNAseq of whole tumor specimens and bulk RNAseq of multiple regions within the same tumor, was to perform more intricate analysis at the level of single

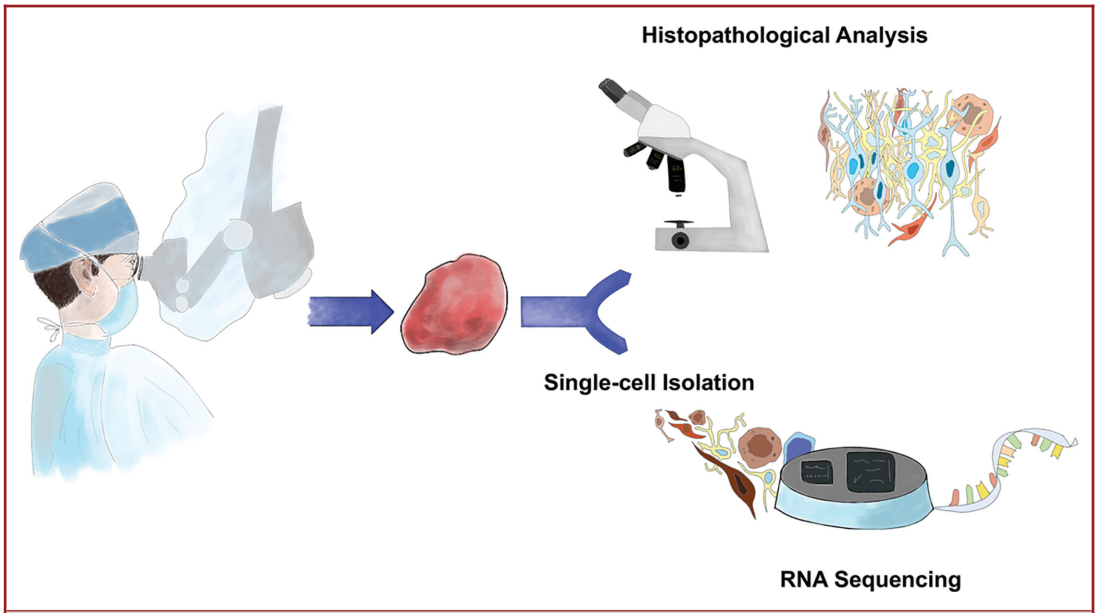

FIGURE 2. *Graphical depiction of sample workflow incorporating scRNAseq into a neurosurgical practice.*

cells. These efforts have been made possible by advances in the realm of single-cell genomics, transcriptomics, and proteomics. A full description of these technologies and their benefits are reviewed elsewhere.[14-16]

Briefly, these single-cell analytic tools overcome key limitations of traditional bulk analytics. They enable discernment of differential gene expression of single cells and also allow for assessment of the relative contributions of cancer cells and noncancerous cells in the TME to overall gene expression profiles.[15,16] scRNAseq is revolutionizing the way we understand malignancies and has the potential to provide fundamental new insights into mechanisms of drug resistance, stemness, and metastasis.[15] It may have particular translational potential in the development of immunotherapies and targeted therapeutics. For these characteristics, scRNAseq was featured as *Science*'s Breakthrough of the Year in 2018.[17]

While not yet a mainstream technology, the neuro-oncology team can expect their clinical workflow to soon incorporate scRNAseq (Figure 2). This entails isolating and profiling single cells and their RNA from fresh tumor at the time of surgery, followed by the same steps as bulk RNA-seq: reverse transcription, amplification, library generation, and sequencing.[18] Currently, widespread clinical adoption of scRNAseq remains hindered by multiple factors.[16,18] scRNAseq is costly and time-consuming. Human and technological expertise are required to analyze complex data and make sense of background noise. Biologically, establishing a milieu that does not interfere with cellular characteristics is time-sensitive and tenuous.[19] Gold-standard techniques and pipelines have yet to be developed.[18] However, an increasing number of institutions are harnessing this technology and beginning to report their experiences.[20]

Ultimately, scRNAseq has transformed the way we study GBM and allowed us to understand its heterogeneity in unprecedented detail. Below, we review seminal studies published to date regarding scRNAseq in human GBM.

"ZOOMING IN" PART 2A: SINGLE-CELL RNASEQ OF GBM TUMOR MASS

Key scRNAseq Studies in GBM

Patel et al[21] performed scRNAseq on 430 cells from 5 primary GBMs in their 2014 study. They found that each of the 5 tumors contained multiple GBM subtypes, each with a dominant Verhaak subtype but with multiple cells that also mapped to other subtypes.[21] Based on bulk expression data, using Verhaak classification, the tumors in their study scored as proneural, classical, or mesenchymal subtypes.[9,21] Using scRNAseq, they found that all tumors had some cells conforming to a proneural subtype regardless of the dominant subtype of the tumor, whereas each of the other subtypes was below detection in at least 1 tumor.[21] However, among individual tumors and across the collective group, they identified four gene expression metasignatures related to hypoxia, complement/immune response, oligodendrocytes, and cell cycle.[21] They also identified a mosaic pattern of expression between individual cells for key cell surface receptors, including *EGFR*, *PDGFRA*, *GFGR1*, *ERBB2*, *ERBB3*, *KIT*, *FZD3*, *NOTCH2*, *EPHA4*, *TGFBR1*, and *GAPDH*.[21] The authors[21] also identified a stem-like compartment distinct from differentiated cells, which paralleled progenitor cells in the developing brain. These cells were particularly resistant to

traditional therapies. Importantly, the authors[21] demonstrated that increased tumor heterogeneity was associated with a dose-dependent decrease in survival; higher levels of intratumor heterogeneity were associated with progressively worse overall survival compared to relatively more homogeneous tumors.

Another key study was performed by Darmanis et al in 2017. Just as prior work with bulk RNAseq distinguished between the tumor core and periphery, Darmanis et al[7] utilized scRNAseq on 3589 cells from the GBM tumor core and peritumoral region in 4 patients. A central component of the study was identifying and characterizing the infiltrating neoplastic cells at the migrating front in the peritumoral region, which is thought to give rise to tumor recurrence. These cells resembled the respective tumor cores from each patient. Additionally, despite the overall heterogeneity among these infiltrating cells, they had a comparable gene expression signature enriched for genes involved in cellular size regulation, energy production, inhibition of apoptosis, cell-cell adhesion regulation, and central nervous system (CNS) development.[7] Darmanis et al[7] concluded similar findings to Patel et al,[21] in that each of the 4 patients' tumors represented an ensemble of cells belonging to multiple Verhaak subtypes.[9]

Venteicher et al[22] performed a third key scRNAseq study on 16 patient samples of isocitrate dehydrogenase (IDH)-A (astrocyte) and IDH-O (oligodendrocyte) gliomas. This study is somewhat beyond the scope of our review, as it is not explicitly focused on GBM, although we note some key findings. The authors[22] found that these two classes of IDH-mutant gliomas were distinguished by particular genetic events and differently composed TMEs, rather than by the existence of distinct progenitor cells; this overturned previous thinking that these different IDH mutants were distinguished by different cell lineages. They also found that increased glioma grade was associated with enhanced proliferation of malignant cells and a larger number of undifferentiated cells.[22]

"ZOOMING IN" PART 2B: SINGLE-CELL RNASEQ OF GBM TME

TME Overview

It is increasingly accepted that clinical management of GBM will necessitate not only targeting the tumor mass itself but also the TME. This TME contains multiple noncancerous cells, including endothelial cells, pericytes, fibroblasts, immune cells, microglia, astrocytes, and neurons.[23,24] The brain is also uniquely insulated by the blood-brain barrier.[24]

The TME is now understood to play a critical role in promoting or suppressing cancer and determining the efficacy of therapeutic modalities.[21] TME components of particular interest are tumor-associated macrophages (TAMs), given their ability to modulate the TME and potential correlations with clinical outcomes.[25] These TAMs are distinguished into two categories: intrinsic/resident brain microglia, whose progenitors migrate to the CNS during early development, and macrophages differentiated from bone marrow-derived monocytes.[26,27] Characterizing individual cells in the TME, including distinguishing between distinct classes of TAMs, has historically proven to be technically challenging. scRNAseq has overcome many of these limitations and enhanced our understanding of the TME. We discuss notable studies in this area below.

Key scRNAseq Studies of GBM TME

Muller et al[28] were the first to use scRNAseq to investigate GBM-derived myeloid cells; their 2017 study provided multiple insights into the heterogeneity and putative roles of distinct TAMs in the GBM TME. They isolated TAMs from patient biopsies and profiled them against macrophages from noncancerous tissue, murine glioma models, and the Ivy GBM Atlas Project.[13] They found that TAMs from peripheral blood and brain-resident microglia had distinct phenotypes and gene signatures: blood-derived TAMs upregulated immunosuppressive cytokines (M2 phenotype) and markers of oxidative metabolism compared to the more pro-inflammatory (M1) brain-resident microglia.[28] Notably, many individual TAMs co-expressed M1 and M2 genes in individual cells, representing both pro-inflammatory and anti-inflammatory, immunosuppressive expression patterns, respectively; this mosaicism paralleled that of the GBM tumor itself.[21] TAMs were also spatially distinct; blood-derived TAMs aggregated in the necrotic tumor core and perivascular regions, while microglia aggregated in the leading edge.[28] Muller et al[28] noted the presence of pro-inflammatory microglia in the leading edge correlating with the peritumoral inflammation that we see clinically. Importantly, their analysis demonstrated that increased expression of blood-derived TAMs was associated with inferior patient survival, while expression of microglial TAM was not. This correlated with their finding (also noted by Venteicher et al[22]) that GBM had higher levels of bone-marrow-derived macrophages than lower-grade gliomas.[28]

The aforementioned scRNAseq study by Darmanis et al also examined the GBM TME; their findings largely corroborate those of Muller et al. Spatially, they showed that the tumor core and peritumoral spaces were preferentially enriched in macrophages and resident microglia, respectively.[7] Cells in the tumor periphery expressed more pro-inflammatory markers, while those in the tumor core expressed anti-inflammatory and pro-angiogenic factors. The authors[7] suggested that these noncancerous cells in the TME may play critical roles in tumor growth, survival, and remodeling the extracellular matrix.

FUTURE DIRECTIONS

scRNAseq is in its nascent stages but clearly has significant potential to shape our understanding and treatment of GBM. Below, we discuss a breadth of future directions of this technology concerning potential clinical and research applications relevant for the neurosurgeon, neuro-oncologist, and radiation oncologist.

CLINICAL IMPLICATIONS

Goals of Operative Management

Great strides in intraoperative care have been made in recent years with improvements in neuro-navigation, intraoperative magnetic resonance imaging, fluorescent labeling with 5-aminolevulinic acid (5-ALA), motor mapping, and awake craniotomy techniques, leading a movement toward extending surgical resection beyond the contrast-enhancing portion of the tumor. This more aggressive approach has been referred to as supratotal resection, and emerging data from studies investigating this approach have shown promising results.[29-33] Extrapolating from scRNAseq studies to date, the field might ultimately move toward more extensive initial resections to remove infiltrating neoplastic cells from the tumor periphery.[7] As a corollary, we postulate that scRNAseq may also inform and indicate the use of emerging ablative techniques, such as laser interstitial thermal therapy (LITT), rather than biopsy alone for some inaccessible tumors.[34] Rigorously studying these operative approaches prospectively may help with answering remaining questions regarding the potential value of supratotal resections and LITT in GBM.[32,33,35]

Immunotherapy

We anticipate that scRNAseq may help address unmet needs related to immunotherapy in GBM, including development of biomarkers, understanding and overcoming treatment resistance, and selectively targeting particular cells in the immune microenvironment.[36,37]

While generally accepted biomarkers for immunotherapy success in cancer include mutational burden and expression of checkpoint ligands,[38] there is a paucity of validated biomarkers in GBM. scRNAseq may facilitate identification of potential biomarkers and subsequently guide GBM immunotherapy through profiling of the immune microenvironment.[39] The breast cancer and melanoma literatures suggest that this may involve identifying and targeting immunosuppressive cell populations.[40,41] For example, in one study prospectively employing scRNAseq to guide treatment for HER2-positive breast cancer, the authors[40] isolated and performed scRNAseq on $CD45^+$ tumor-infiltrating cells and subsequently discovered that treatment-responsive tumors contained more T and NK cells, while treatment-resistant tumors contained clusters of cells with signatures (ie, elevated expression of *Arg1* and *Xbp1*) resembling immunosuppressive immature myeloid cells. These findings enabled them to overcome this initial treatment resistance and immunosuppressive microenvironment with sequential combination therapy regimens involving specific targeting of immunosuppressive cell populations.[40] A related future direction could involve specifically targeting the immunosuppressive TAMs derived from peripheral blood, rather than brain-resident microglia, to augment the host immune response to GBM.[28] Other immunotherapy-related efforts may involve identifying signatures of treatment resistance in shared subgroups of cells[42] and describing specific T-cell states with prognostic value.[43]

Furthermore, increasing evidence suggests that intratumor heterogeneity may be an essential factor determining immune surveillance and response to therapy, independent of mutational burden.[44-46] scRNAseq may facilitate better understanding of this heterogeneity. It may also be used to determine which tumor antigens are expressed on the most clones within a tumor, which could guide selection of appropriate immune therapies.[45,47]

Radiotherapy

It is well established that many recurrent cases of GBM arise from regions beyond the resected gadolinium-enhancing portion of GBM.[48,49] This failure, despite adjuvant radiation and chemotherapy, heralds a need for re-evaluation of the understanding of single cells in the peritumoral brain zone.[49] Indeed, we now know that GBMs have stem cells that are particularly radioresistant.[50] While conclusions from these studies are not yet clinically actionable, we anticipate that additional knowledge of single cells in the tumor margins may eventually facilitate more potent and improved radiotherapy in this problematic area.

Personalized Medicine

scRNAseq will likely play an essential role in the shift toward personalized medicine within oncology. These efforts are already underway; in the NCT Neuro Master Match (N2M2) pilot study, Pfaff et al[51] performed real-time molecular profiling of patients' tumors, in which each patient represents his/her own personalized study. Similar efforts may allow for individualized treatment planning as well as better prognostication, perhaps augmented with new scoring systems.[52] Surgically, careful consideration can be given to gross total resection vs supratotal resection given individual patient circumstances. Individual patient-level scRNAseq data might also allow for the withdrawal of ineffective or unnecessarily toxic medications that are likely to be ineffective based on tumor genetics.

RESEARCH IMPLICATIONS

Modeling Tumor Heterogeneity

The depth of understanding that scRNAseq provides has great implications for future research. First, it may facilitate the development of better preclinical models, including cell, animal, and patient-derived xenografts, which more accurately represent the heterogeneity of human GBM. This may subsequently increase the likelihood of laboratory breakthroughs translating to successful therapies in humans. We might also better understand mechanisms of drug resistance and more rigorously test combination therapies in the laboratory to overcome this resistance. For example, building upon the demonstrated intratumor heterogeneity reported through scRNAseq methods, Teng et al[50] demonstrated an aggressive subpopulation of patient-derived GBM cells with high stem-like properties that resisted

adhesion and differentiation in Vitro; these cells were found to be resistant to radiation and targeted therapies. With continued assistance of scRNAseq, future research efforts in GBM may become more fruitful by appropriately modeling tumor heterogeneity and honing in on the specific cell subpopulations that prevent disease eradication and give rise to recurrence.

Recurrent GBM

An important area for future research will be the application of scRNAseq to recurrent GBM. Such work might involve scRNAseq of naïve GBM, treated GBM, and recurrent GBM from the same patients over time. Glimpses of this work have shown promise, with one patient's tumor harboring three mutated genes within single cells involved in the RAS/Guanine nucleotide exchange factor guanosine triphosphate-dependent signaling pathways.[53] This work might allow prediction of mutations that will arise from primary to recurrent tumors and may help identify targetable drivers of tumor recurrence. A significant obstacle that must be overcome in analyzing recurrent GBM tissue is accounting for technical variability and confounding factors, including apoptotic cells and the low yield of viable cells to analyze upon tumor recurrence.[54]

Clinical and Virtual Drug Trials

A clear clinical implication of GBM tumor and TME heterogeneity—such as mosaic expression of surface receptors and TAM markers—is the necessity for combination therapy regimens that effectively target all cells within a given tumor while thoughtfully targeting particular cells in the TME.[21] Other therapeutic avenues might involve specifically targeting the infiltrating GBM cells at the tumor periphery and attempting to promote the differentiation of stem-like cells in order to arrest tumor growth.[7,22] These efforts may become the subject of forthcoming clinical trials attempting to personalize GBM therapies. While the application of scRNAseq to large clinical trials has not yet been explored, we anticipate that it may ultimately allow for the creation of better-matched cohorts, as well as identifying appropriate trials and interventions for each patient based on the cellular and molecular signature of their individual GBM.[55]

Another exciting avenue might incorporate advances in computer science and machine learning, such as the virtual drug trial developed by Barrette et al.[56] Their simulation-based approach integrates patient-specific data with known data about various cancer pathways to prioritize and virtually test different drug candidates and combinations in GBM. With the increased precision afforded by scRNAseq, such trials could potentially assist the neuro-oncologist in choosing appropriately guided therapies for each patient.

scRNAseq as a Complement to Bulk RNAseq

Bulk RNAseq will likely continue to play a large role in future GBM research due to aforementioned financial and practical limitations of routinely using scRNAseq. However, bulk sequencing will likely be increasingly used in a complementary fashion with scRNAseq.[15,16] These data might then be combined with other single-cell resolution approaches, such as phosphoproteomics, to better characterize the contribution of individual cells in GBM.[57]

CONCLUSION

GBM is a profoundly heterogeneous cancer, which complicates research and clinical efforts aimed at improving its rapidly progressive, lethal course. scRNAseq is a revolutionary tool that has facilitated unprecedented, high-resolution characterization of this heterogeneity within GBM and its TME. This work is likely to shape clinical practice and accelerate future research advances in GBM to more effectively target each individual cancer. While still in its nascent stages, scRNAseq appears to have the potential to improve GBM patient outcomes, and we can expect it to be increasingly incorporated into the clinical and research ecosystem surrounding GBM in the years ahead.

Disclosures

The authors have no personal, financial, or institutional interest in any of the drugs, materials, or devices described in this article. Dr Bettegowda is a consultant for Depuy-Synthes.

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