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Single-Cell RNA-Sequencing in Glioma

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Abstract

Purpose of Review—In this review, we seek to summarize the literature concerning the use of single-cell RNA-sequencing for CNS gliomas.

Recent Findings—Single-cell analysis has revealed complex tumor heterogeneity, subpopulations of proliferating stem-like cells and expanded our view of tumor microenvironment influence in the disease process.

Summary—Although bulk RNA-sequencing has guided our initial understanding of glioma genetics, this method does not accurately define the heterogeneous subpopulations found within these tumors. Single-cell techniques have appealing applications in cancer research, as diverse cell types and the tumor microenvironment have important implications in therapy. High cost and difficult protocols prevent widespread use of single-cell RNA-sequencing; however, continued innovation will improve accessibility and expand our of knowledge gliomas.

Keywords

Single-cell; RNA-sequencing; Glioma

Introduction

Single-cell RNA-sequencing (scRNA-seq) provides an opportunity to access information about cellular biology at unprecedented resolution. scRNA-seq allows the user to analyze the transcriptome from individual cells as reviewed in Wang et al. [1], including single-cell detection of novel transcripts [2, 3], developmental changes [4•], alternative expression [5], splicing variants [6], or underlying mutations [7]. This provides a unique opportunity to determine the interplay between intrinsic cellular processes and environmental stimuli. Bulk RNA-seq averages the expression profiles of potentially diverse cells, leading to loss of contribution from heterogeneous or rare populations. The resulting sequences from scRNA-

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seq can be compared between cells, depicting specimen heterogeneity or identification of a rare population of cells.

Gliomas, the most common primary central nervous system (CNS) tumors in adults, are known for their heterogeneity and rapid clinical progression [8]. Histologically, identical tumors can have varied underlying mutations and can, therefore, respond very differently to the same treatments [9]. Personalized genomic dissection of CNS tumors will better enable the identification of actionable targets for molecularly guided therapies [9]. Thus far, over 70 oncogenetic variants have been identified in CNS tumors through conventional methods [10] with some gene expression profiles linked to clinical outcomes [11, 12]. Molecular marker identification in glioma has defined clinically relevant sub-classifications. For example, the presence of mutations in IDH1 and IDH2 are consistent with an improved prognosis [13]. Uncovering and harnessing this tumor heterogeneity will allow a more personalized medical response in the oncologic treatment of CNS tumors.

Widespread implementation of scRNA-seq has been difficult due to difficult isolation procedures, cost, and complex bioinformatics interpretation. The first step in the process involves creating a single-cell suspension by dissociating target tissue samples, without disrupting or degrading their gene expression patterns. Individual cells are commonly isolated using microfluidic devices [14–16], manual picking [17–19], or fluorescence-activated cell sorting [20–22]. Single-cell suspension, isolation, and collection are often time-intensive techniques and techniques vary based on tissue of interest. scRNA-seq involves isolating and lysing single nuclei, reverse transcription, cDNA amplification, and transposase Tn5-based fragmentation for library sequencing preparation [7]. As technology improves and cost decreases [23], scRNA-seq utilization will increase, improving our understanding of heterogeneous tissues.

Single-Cell RNA-Sequencing of Normal Brain Tissue

scRNA-seq is well suited to disentangling the brain's cell diversity and rare subpopulations [1]. Bulk RNA-seq has created high quality databases using human and mouse brain tissue instrumental in our understanding of the main brain cell subtypes (http://web.stanford.edu/group/barres_lab/brainseq2/brainseq2.html) [15–17]; however, the signal from rare brain cell populations may not be detectable with this method. In a study of 3005 single cells from normal mice brains, Zeisel et al. identified 47 distinct subclasses of cells, including 16 subclasses of interneurons and 7 subclasses of pyramidal cells, in the somatosensory cortex and CA1 hippocampus [14]. Oligodendrocytes that were thought to be all the same were divided into six different classes. This level of diversity has been identified in other cellular types and brain regions, including the primary visual cortex, dentate gyrus, striatum, corpus callosum, amygdala, hypothalamus, zona incerta, SN-VTA, and dorsal horn [4•, 14, 24–27]. In an analysis of 466 normal human adult and fetal temporal lobe cells, Darmanis et al. found that scRNA-seq was capable of classifying cells as astrocytes, oligodendrocytes, oligodendrocyte precursor cells (OPCs), neurons, microglia, and vascular cells [28•]. Although most scRNA-seq studies thus far in neural tissue have been descriptive [29] (Table 1), scRNA-seq is an important technique to define the functional diversity within the brain.

Single-Cell RNA-Sequencing of Glioma

Overview

In adults, there are three main categories of gliomas, determined by genetic and histologic features: glioblastoma (GBM), astrocytoma, and oligodendroglioma. GBM is most frequently IDH-wild-type, while IDH1 and/or IDH2 mutations are found in astrocytoma, and oligodendroglioma [35]. Gain of chromosome 7 and loss of chromosome 10 are the earliest and most common genetic alterations in GBM analyzed by bulk RNA-seq [36]. Similarly, these genetic alterations have been confirmed in individual tumor cells using scRNA-seq [30, 31, 33••]. Astrocytomas with IDH mutations (IDH-A) frequently have ATRX and TP53 mutations, while oligodendrogliomas (IDH-O) have mutations in the TERT promoter and loss of chromosome arms 1p and 19q [37]. Venteicher et al. found that most of the variation in expression by malignant cells in IDH-1 mutant tumors is attributable in the aforementioned genetic events, such as loss of chromosome arm 1p when scRNA-seq was employed [34••].

Bulk RNA-seq fails to accurately define the expression profiles of the diverse cell subpopulations in glioma, leading to an underappreciation of heterogeneity and a misclassification of tumors. Verhaak classifications in GBM defined tumors as proneural, neural, classical, or mesenchymal, predominantly using differences in bulk gene expression of EGFR, NF1, PDGFRA, and IDH1 [38]. scRNA-seq has highlighted inconsistencies in the model as GBMs are likely mixtures of these classifications [31, 32•]. Genetic heterogeneity in glioma is attributed to transcriptional variation in cell signaling, proliferation, the complement system, immune response, and hypoxia in the malignant and non-malignant cells comprising the tumor. In an analysis of 430 cells from 5 GBM samples, Patel et al. reported that cells from the same tumor sample varied in correlation ($r = 0.2$ to $r = 0.7$), highlighting intratumoral diversity [30]. The documented heterogeneity could potentially explain classification switching seen in recurrent GBM following treatment. Similarly, individual cells obtained from IDH-mutant tumors contain mixtures of both astrocytes and oligodendrocytes [30, 34••].

Tumor heterogeneity is further demonstrated through mosaic expression of target genes. Receptor tyrosine kinases (RTKs) such as EGFR, PDGFRA, and PDGFA are frequently amplified and rearranged in GBM [39, 40]. However, tyrosine kinase inhibitors (TKIs) have shown limited efficacy. Single-cell genomics have highlighted variable expression of RTKs in GBM. Cells within GBM express different TKI resistant EGFR and PDGFRA variants [31]. Additionally, individual tumor cells may co-express multiple EGFR variants [33••]. Interestingly, most neoplastic cells do not express CD274, PDCD1LG2, CD80, or CD86, which suggests that checkpoint inhibitors, therapeutics directed against these targets, could have limited efficacy in GBM [33••]. scRNA-seq revealed inconsistent expression of drug targets which may have contributed to treatment failure.

Spatial localization of individual cells within glioma accounts for a portion of their heterogeneity. Cells positioned in the center of the glioma are likely to be hypoxic while cells on the edge and periphery of the tumor are well perfused. Darmanis et al. confirmed the magnetic resonance image-guided surgical resection of tumor core ($N = 2343$) and

surrounding (N = 1246) cells using classical hypoxic genes [33••]. Additionally, the genetic expression of neoplastic cells surrounding the tumor core and malignant cells within the core differs. Peripheral neoplastic cells expressed high levels of PRODH, FGFR3, and LMO3, involved in proline catabolism for ATP production, cell survival signaling and inhibition of TP53-mediated apoptosis, respectively [33••]. Müller and colleagues described an infiltrating phenotype for neoplastic cells on the tumor periphery, overexpressing genes involved in cell survival (survivin) and genomic instability (Aurora B kinase), as well as genes involved in downstream cell adhesion [31]. Notably, these findings involved 63 cells from a single patient. Tumor cell localization contributes to heterogeneity and has implications in targeted therapeutics.

Proliferating Glioma Subpopulations

Most of the cells in glioma are non-proliferative; however, a population of proliferating, undifferentiated progenitor cells has been hypothesized as the drivers of GBM spread and recurrence following treatment [41]. Darmanis et al. found that 7.7% (80/1029) of neoplastic cells in the core and 1.6% (1/62) neoplastic cells in the tumor periphery proliferate in GBM [33••]. These proliferating cells resembled oligodendrocyte progenitor cells (OPCs), which are typically found in the developing brain. Oligodendrogliomas were reported by Tirosh and colleagues to have two distinct differentiated non-proliferative linages, representing an astrocyte and oligodendrocyte population. Notably, a third population of undifferentiated proliferating cells (~ 10% of 4347 cells) resembling neural progenitor cells (NPCs) were found instead of OPCs in all their tumor samples [4•]. Similarly, Venteicher et al. found that IDH-mutant cells shared the same developmental hierarchy, each consisting of non-proliferating astrocytic and oligodendrocytic lineages, as well as proliferating undifferentiated cells that resembled NPCs [34••]. Comparing the undifferentiated populations from IDH-mutant tumors revealed high similarity, indicating the possibility of a shared cell of origin for the tumor types [34••]. The OPC-like and NPC-like cells from GBM, and IDH-mutant tumors expressed neurodevelopmental transcription factors, such as SOX2, SOX4, SOX9, SOX11, NFIA, and NFIB at high levels [4•, 30, 33••, 34••]. Top expressed genes involved in neurogenesis were, ASCL1, CHD7, CD24, POU3F2, BOC, and TCF4 [4•, 30, 33••, 34••]. Single-cell expression analysis has consistently supported NPCs and OPCs as the drivers of tumor growth and implies that induced differentiation of these cells could be an effective therapy. This potentially important treatment target could not be identified using bulk—RNA-seq analysis alone.

Immune Cells in the Tumor Microenvironment

The tumor microenvironment (TME) is composed of extracellular matrix, fibroblasts, vascular cells, neurons and immune cells. Darmanis et al. found that only 44% of tumor core-originating cells segregated to neoplastic cell clusters [33••]. Analyzing 6 oligodendroglioma tumor samples using scRNA-seq revealed that only half of the differentially expressed genes identified by bulk RNA-seq were expressed by malignant cells, suggesting a significant influence from the TME [34••]. Most of the expression differences in the TME were microglia/macrophage-specific genes and neuron-specific genes. These findings indicate that the TME may represent a significant portion of bulk RNA-seq analysis.

Immune cells in the TME are uniquely positioned to influence glioma behavior and tissue organization. Most immune cells within the TME are macrophages and mircroglia (> 95%), while the remaining cells are primarily dendritic cells (~ 4.5%) [32•, 33••]. Macrophages were reported to be preferentially found in the tumor core (813 macrophages/365 microglia) and microglia were located in the surrounding cells (85 macrophages/574 microglia) [33••]. Pro-inflammatory markers were expressed in the tumor periphery (IL1A/B), while more anti-inflammatory (IL1N1) and pro-angiogenic (TGFBI) factors were expressed in the tumor core by macrophages and microglia [33••]. Although there seems to be a distinction in the gene expression of macrophages and microglia in glioma, the differences occur along a spectrum. These results suggest that the properties of the TME influence immune cell gene expression despite cell origin [34••].

scRNA-seq has begun to define the complex interactions between TME immune and neoplastic cells. For example, Wang et al. reported that NF1 deficiency was associated with increased tumor-associated macrophage/microglia infiltration and that the mesenchymal subtype of GBM was associated with increased M2 tumor promoting macrophages [32•]. However, a causal relationship has not been explored for either of these findings to date. Higher-grade IDH-mutant tumors were preferentially associated with macrophage-like expression states in the TME [34••]. These findings are likely due the high degree of angiogenesis and permeability of the blood brain barrier found in high grade lesions, although more research must be done to further define this relationship. Finally, IDH-A tumors have been reported to contain more immune cells than IDH-O tumors and this difference was not accounted for by tumor grade or endothelial cell contamination [34••]. Taken together, these findings highlight the need for more comprehensive studies exploring TME immune cell and neoplastic cell relationships.

Conclusion

Bulk RNA-seq expression profiles have been instrumental in our initial understanding of brain biology and glioma, but provide limited insight into tissue heterogeneity and identification of rare cellular subtypes. scRNA-seq has revealed complex tumor heterogeneity and expanded our understanding of cancer progenitor cells and TME interactions. Significant cost and technical challenges are current barriers to wide spread implementation. A better understanding of the molecular features of CNS tumors through scRNA-seq will aid in the development of novel treatment strategies.

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Table 1

Significant contributions to our understanding of brain diversity through scRNA-seq

Year	First author	Tumor type
2014	Patel [30]	Human GBM
2015	Shin [24]	Mouse normal brain
2015	*Darmanis [28•]	Human normal adult and fetal brain
2016	Müller [31]	Human GBM
2016	*Tirosh [4•]	Human IDH-O
2017	*Wang [32•]	Human derived GBM neurospheres
2017	**Darmanis [33••]	Human GBM and normal surrounding brain
2017	**Venteicher [34••]	Human IDH-A and IDH-O

GBM glioblastoma multiforme, *IDH-A* astrocytoma, *IDH-O* oligodendroglioma

* denotes an important reference

** denotes a very important reference

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