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The Dynamic m⁶A Epitranscriptome in Glioma Stem Cell Plasticity and Function

David Karambizi and Nikos Tapinos

Abstract

Glioblastoma multiforme is one of the most aggressive tumors of the central nervous system. The current standard-of-care includes maximal resection followed by chemotherapy, radiation and more recently, tumor treating fields (TTFs). Despite this multimodal approach, glioblastoma remains refractory to therapy. Glioblastoma resistance, recurrence and malignancy are believed to be driven by a subpopulation of glioma stem cells (GSCs) within the tumor bulk which are characterized by the retention of self-renewal potential as well as the capacity to recapitulate tumor heterogeneity. Within the dynamic intratumoral niche, GSCs demonstrate a high degree of cellular plasticity, reversibly interconverting between stem-like states and more differentiated states as a result of environmental cues/signaling fluctuations. Such plastic adaptive properties are mostly driven by multiple dynamic, reversible epigenetic modifications. We posit that reversible post-transcriptional methylation of RNA transcripts at the m⁶A position may be one such regulatory mechanism employed by GSCs to efficiently maintain plasticity and adaptive phenotypic transitions. In this section, we discuss the concept of cellular plasticity, introduce dynamic m⁶A epitranscriptomic mechanisms as potential key regulators of GSC plasticity and finally propose epigenetic based therapeutics as a mean of attenuating glioblastoma plasticity to improve patient outcome.

Keywords: glioma stem cell, plasticity, epigenetic landscape, epitranscriptome, cellular states, glioblastoma

1. Introduction

Glioblastoma is one of the most lethal malignant tumors of the central nervous system. Its treatment involves maximal resection followed by chemotherapy, radiation and tumor treating fields [1]. Despite this multimodal approach, GBM remains uniformly lethal, with a median survival of 15 to 16 months [1]. Histologically, GBM presents as a heterogenous mass with multifocal necrosis, hypervascularization, hemorrhage, pleiomorphic cells with notable mitotic activity and pseudopalisading nuclei [2, 3]. Recent advances in whole genome sequencing allowed for better GBM characterization to compliment current medical knowledge.

The Cancer Genome Atlas (TCGA) initiative generated DNA, RNA and methylation sequencing data on multiple GBMs and lower grade gliomas [4], shedding light onto GBM specific structural, mutational and methylation alterations. It was

shown that NF1, IDH, PDGFRA and PARK2 were mutated and that AKT3 and EGFR were amplified in GBMs [5, 6]. Additionally, the vast majority of GBMs were shown to activate the RB, p53 and RTK/RAS/PI3K pathways [5]. Using tumor gene expression signatures, patients could be categorized into discrete subtypes, namely mesenchymal, proneural and classical [6]. However, subtyping did not directly relate to long term survival [7]. Tendencies towards survival were only observed when the data was restricted to patients with lowest simplicity score [7].

The TCGA derived data supplied useful information, but it simultaneously raised new questions. First, it was noted that 8% of the samples did not discretely fit within defined TCGA subtypes, but instead scored for multiple subtypes [6–8]. Second, tumors were shown to undergo subtype switching following recurrence [9]. Third, even with low mutational burden, GBM exhibited significant intra and inter tumoral heterogeneity. GBM's aggressiveness and recurrence is believed to be driven by a small subpopulation of stem like cells within the tumor niche [10–13]. These cells, generally referred to as glioma stem cells (GSCs), possess the ability to self-renew and can fully recapitulate the tumor bulk with fidelity to parental tissue properties following xenotransplantation [14]. Recent developments have helped to catapult GSCs at the nexus of GBM tumorigenesis. It has been shown that the adult human brain is not an entirely post-mitotic tissue and to possess specific regions with an enrichment for cells with stem like properties or neural stem cells (NSCs) [14, 15]. Interestingly, NSC markers such as CD133 and Nestin are frequently expressed in GSCs [16]. Such homology raised questions on GSCs relation to NSCs. Thus, “the cell of origin” theory emerged. The theory posited that GSCs, which are mutated NSCs are the cells of origin of GBM. Spatial studies demonstrated that GBMs exhibited a growth bias for the subventricular zone (SVZ), a region known to be enriched with NSCs [14]. Furthermore, multiple studies showed that *de novo* GBM tumorigenesis could be achieved by inducing tumor initiating mutations within the SVZ [8]. Together, these findings cemented GSCs as initiators and drivers of GBM, hence placing them center stage as key targets in GBM therapy. However, most therapies targeted at GSC continue to fail, likely due to GSCs' high adaptability potential and tendency to continuously fuel tumor niche dynamic heterogeneity by undergoing reversible multilineage differentiation.

The aforementioned complex cell dynamics likely rely on coordinated genetic and epigenetic processes. Here, we focus on epigenetic processes, more specifically post transcriptional chemical decorum on mRNA adenosine or mRNA m6A. This chemical modification has widely been explored in dynamic processes ranging from neurogenesis, memory formation to various pathophysiological processes including cancers [17–19]. We discuss what is known at the m6A/plasticity interface in GBM and finally postulate/propose ways in which epitranscriptomics can function as a predictive or therapeutic tool to affect clinical outcome.

2. Cellular plasticity in glioblastoma

GBM exhibits a high degree of intertumoral and intratumoral heterogeneity. Such heterogeneity is sustained by constant, dynamic interconversion between cellular states. Differentiated glioma stem cells (DGCs) undergo spontaneous de-differentiation to primordial states or back to GSCs and vice versa in response to fluctuating microenvironmental cues [20–23]. It is likely that this tumor hijacks highly conserved genetic and epigenetic programming generally associated with stemness multipotency and early embryonic development in order to rapidly adapt to and evade various therapeutic strategies. Therefore, glioma cancer cells leverage such plasticity to maintain an adaptive, shifting cell state population equilibrium

that is not amenable to therapy. For example, radiotherapy and temozolamide induce adaptive, spontaneous de-differentiation of DGCs to GSCs, thereby increasing and replenishing the cancer stem pool [24]. Such a shift in cell population distribution towards increased stemness forcibly translates to a more refractory tumor organ.

Recent work shows that GSCs clones are able to readily undergo reversal phenotypic transition between clonal populations [21]. The authors also demonstrated the reversible nature of the cellular equilibria assumed by GSCs in the face of hypoxia as the cells return to a naïve, pre-hypoxia exposure following normoxia [21]. In a fashion reminiscent of the Waddington landscape, GSCs inherently possess a high cellular plasticity potential, thus exist in thermodynamically poised cellular states, and can adaptively differentiate to assume multiple population equilibria in response to external perturbation [21]. These rapid processes entail myriad cellular epigenetic regulatory mechanisms, one of which is the dynamic regulation of m⁶A.

3. The epitranscriptome in glioblastoma

Currently, there are over 170 possible chemical modifications on RNA species [25]. The majority occurs on highly abundant non-coding RNA species such as rRNAs, tRNAs and snRNAs and consequently influence RNA stability and RNA secondary/tertiary structure [26]. Most of these modifications are challenging to study in mRNA due to their sparsity and relatively higher abundance in rRNA and tRNA, hence imposing a detection problem in coding RNA [25, 27]. Conversely, N⁶-methyladenosine (m⁶A) is highly enriched in mRNA, but sparse in rRNA and absent in tRNAs. The occurrence of m⁶A on mRNA and its effector role on mRNA stability were established in the 1970s [28, 29]. Since, a set of complexes responsible for 1) placement of m⁶A on transcripts (m⁶A methyltransferases or “writers”) 2) removal of m⁶A (m⁶A demethylases or “erasers”) 3) “interpretation” or effector function of m⁶A marks (readers) have been identified. Readers include the YTH domain containing YTHDF1-F3 and YTHDC1-C2. YTHDC1 and YTHDC2 bind methylated nuclear transcript, while YTHDF1, YTHDF2 and YTHDF3 bind methylated cytoplasmic transcript [30–35]. Methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14) and Wilm’s-tumor-1-associated protein (WTAP) form a multimeric methyltransferase complex responsible for m⁶A mark transcript placement [36–38]. The removal of m⁶A is mediated by the fat mass-and-obesity-associated protein (FTO) and alkylation repair homolog protein 5 (ALKBH5) [39, 40]. The discovery of these m⁶A RNA demethylases implied possible reversibility or dynamism inherent to the epitranscriptome. Once placed on transcripts, m⁶A has been shown to modulate mRNA stability, splicing and translation and thus ultimately influence gene expression kinetics and outcome [32–34]. Following the refinement of m⁶A detection techniques, m⁶A has been widely studied in physiologic processes, such as early development, and in patho-physiologic processes, ranging from psychiatric disorders to cancers. Here, we focus our attention to the glioblastoma/m⁶A interface as it pertains to plasticity.

3.1 m⁶A writers in glioblastoma

The most common way the m⁶A code has been probed in GBM is via enzymatic inhibition or transcript level perturbation of the m⁶A machinery. The majority of works on the role of writers in GBM suggest an oncogenic role for METTL3/METTL14. The methyltransferase METTL3 has been shown to be essential for sustenance of GSCs, radioresistance and GBM oncogenic signaling [41–44]. Yet, METTL3 and

METTL14 have been shown in an overexpression-based study to reduce GSCs tumorigenicity and stemness potential, suggesting a potential tumor suppressive function [45]. The reasons for these discrepancies pertaining to m6A writers are unclear and necessitate additional clarifying studies. Ultimately, these results could possibly reflect GBM heterogeneity/complexity and hence dissuade against generalizations on m6A in GBM.

3.2 m6A erasers in glioblastoma

So far, the known m6A erasers exhibit oncogenic tendencies in GBM. Inhibition of FTO demethylase activity has been shown by two independent groups to inhibit stemness propensity in GSCs [45, 46]. In another study, ALKBH5 was shown to be highly expressed in GSCs and functioned to promote tumorigenicity via FOXM1 transcript stabilization [47].

3.3 m6A readers in glioblastoma

The functional role of the YTH readers in GBM had been unknown until very recently. Two recent studies show that YTHFD2 promotes GBM aggressiveness, albeit through different proposed mechanisms [48, 49]. One study finds that YTHDF2, though previously shown to destabilize transcripts, does however stabilize *MYC* and *VEGFA* transcripts in GSCs in an m6A-dependent manner [48]. The other study shows that the EGFR/SRC/ERK pathway functions to stabilize YTHDF2 via protein phosphorylation and YTHDF2 consequently destabilizes transcripts implicated in cholesterol dysregulation and invasive GBM growth [49]. Again, these differences may suggest context dependence given GBM's high levels of heterogeneity and plasticity.

Summary of the role of various components of the m6A RNA methylation machinery in glioblastoma is presented in **Figure 1**. Though hinting at plasticity, most of these studies do not explicitly determine m6A dynamics in the context of GBM cell state transition.

3.4 Role of m6A in cellular plasticity in glioblastoma

Recent findings in neuroscience pertaining to neurogenesis and gliogenesis emphasize the centrality of m6A in dictating cell fate/state specification and plasticity events during early brain development. Stem cells of the nervous system, known as radial glia cells (RGCs) or neural progenitor cells, which are responsible for neurogenesis and gliogenesis, show m6A dependency [50]. As per one study, conditional KO of *Mettl3/Mettl14* in embryonic mouse brain resulted in premature activation of later stage differentiation specific transcripts that are normally kept low in RGCs [50]. Consequently, m6A depleted RGCs could not undergo appropriate multilineage differentiation and expectedly formed abnormal brain tissue [50]. Another study shows that the process of glial specification relies on m6A [51]. Depletion of *Prrc2a*, which is a gene coding for the Olig2 stabilizing m6A reader PRRC2A, results in hypomethylation and cognitive defect secondary to Olig2 transcription factor destabilization [51]. These studies demonstrate the key role of the m6A code in driving cell fate specification, differentiation and hence plasticity via transcriptional regulation during neurogenesis. From these data, a corollary can be drawn that GSCs, which are mutated NSCs, could exhibit significant m6A dependence during differentiation, de-differentiation, tumorigenesis and in response to external perturbation such as radiation and chemotherapy. However, this m6A/plasticity axis in GSCs and GBM niche remains poorly understood. Recently, we

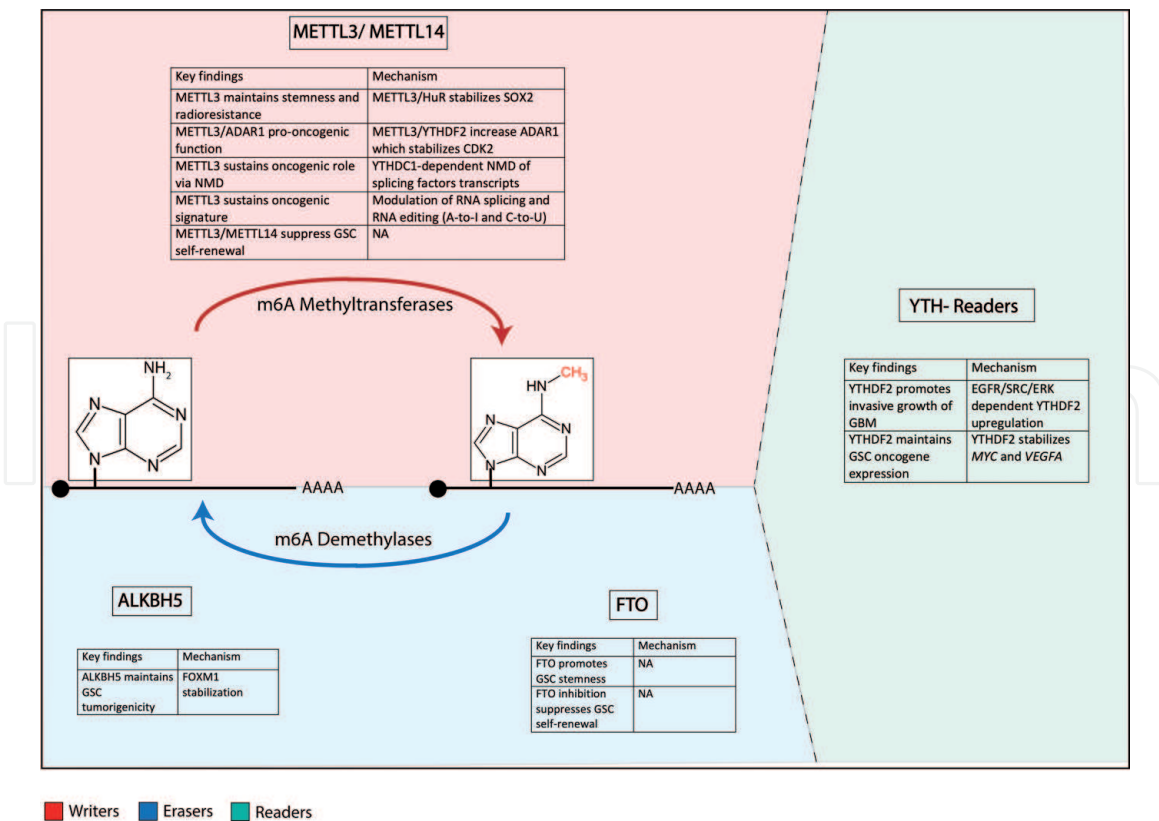


Figure 1. Summary of key findings for the role of the various components of the m⁶A machinery in glioblastoma.

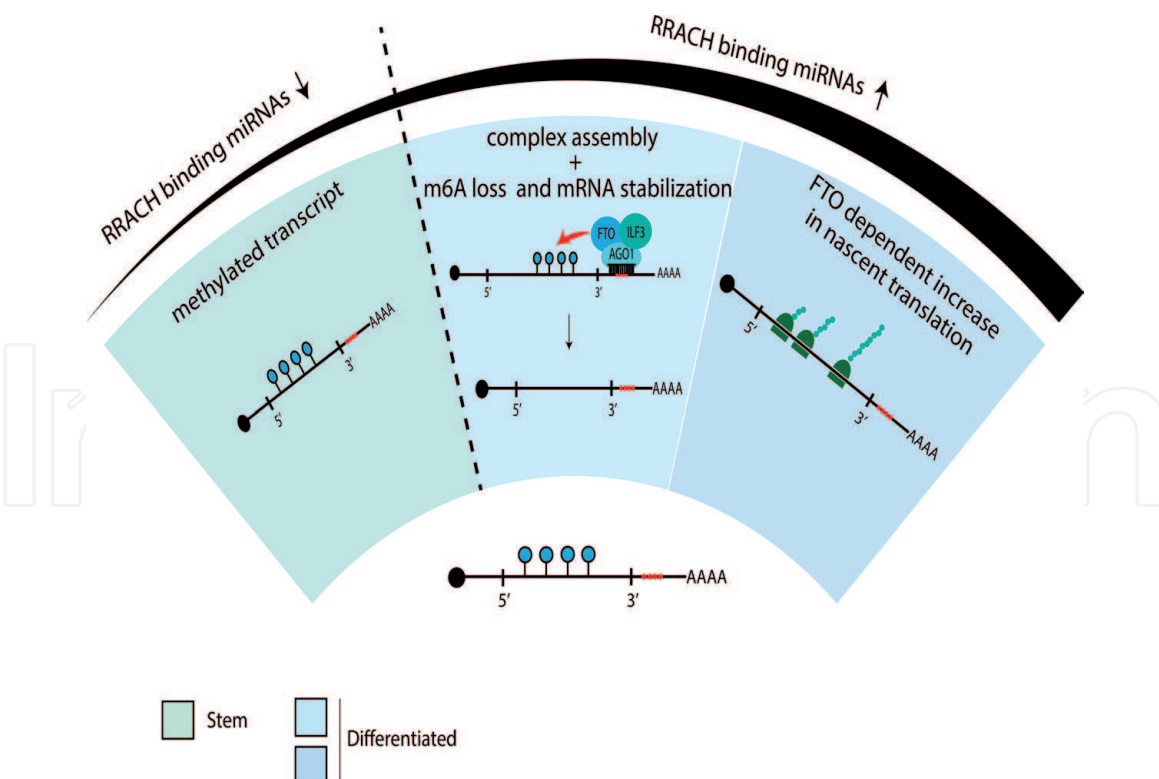


Figure 2. miRNAs direct FTO/AGO1/ILF3 complex on RRACH m⁶A motifs to induce RNA demethylation and increase in nascent translation during the transition of GSCs to differentiated progenies.

performed an integrated whole genome meRIP-seq, RNA-seq and ribo-seq analysis in three patient-derived GSCs and differentiated progenies [52]. This allowed for the interrogation of transcriptional, epitranscriptional and translational kinetics during

cell state transition [52]. In the study, we deliberately avoid m6A machinery perturbation and simply attempt to unravel what happens in one of the most basic process of GSCs plasticity (differentiation) in the context of m6A dynamics. We discovered that a set of clinically relevant transcripts which experience the greatest increase in translation efficiency during differentiation also show significant loss in m6A peaks. This pattern occurred independently of glioblastoma subtypes. We found that these common, highly translated transcripts during GSC differentiation share a consensus m6A motif (the RRACH motif) that overlap a specific set of miRNA sequences. In addition, we discovered a corresponding striking increase in expression of some of these miRNAs with GSC differentiation. Subsequently, we asked whether these findings implicate miRNA at the m6A/translation interface during differentiation. Through a series of mechanistic studies, we propose a mechanism whereby miRNAs can facilitate the formation of a transcript stabilizing FTO-ILF3-AGO1 complex. This results in more efficient association with the ribosome, thus promoting an increase in nascent translation (**Figure 2**).

4. The epitranscriptome as a therapeutic target in glioblastoma

Though in its infancy, the field of epitranscriptomics holds significant promise for the development of novel epigenetic therapies against GBM. Currently, strategies for targeting the m6A machinery in glioblastoma are directed at the inhibition of enzymatic activity [45, 46]. Recently, targeting of the m6A erasers as well as YTHDF2 have shown some encouraging results for GBM treatment [48]. Specifically, it was shown that high levels of YTHDF2 correlate with increased sensitivity to Linsitinib, an inhibitor of the YTHDF2 downstream effector IGFBP3 [48].

Another emerging avenue is the fusion of m6A machinery components to RNA targeting CRISPR complexes [53, 54]. The deployment of the m6A machinery-CRISPR complex can allow for the specific activation or deactivation of specific transcripts via m6A manipulation [56, 57]. The safe and reversible target specific stabilization or destabilization of coding and/or non-coding RNA species represents an exciting frontier in the development of RNA based therapeutics.

However, these findings leave more avenues for inquiry. For example, what role does the epitranscriptome play in de-differentiation of GSCs, in therapeutic evasion and in microstate transitions of GSCs and differentiated progenies? Are there specific “m6A codes” associated with specific cellular microstates? And how do m6A processes work in synergy with other cellular machineries such as miRNAs, long non-coding RNAs, or well established GBM tumor promoting/suppressing signaling pathways to maintain plasticity? Are m6A dynamics driving or secondary events in GBM microstate transitions? Evidently, more work needs to be done to probe the m6A/plasticity interface in GSCs in order to aid in the discovery of novel epigenetic therapies targeting GSC plasticity.

5. A systems approach towards an epigenetic landscape in glioblastoma

Recent advancements in single cell RNA sequencing, which include integrated single cell multi-omics analysis, as well as the application of novel algorithms such as pseudotime and RNA velocity have allowed for better characterization of the dynamics within the heterogenous GBM tumor niche [21, 55, 56]. Initial single cell analysis demonstrated that GBM cancer cells exist in a cell state continuum

with polarization towards specific fates [55]. Additionally, projection of single cell transcriptomics onto a fetal neurodevelopmental roadmap identified previously unidentified glioma stem cell properties and established GSC at the apex of the glioblastoma tumor hierarchy [21]. These rapidly cycling apical progenitor cancer stem cells were found to have a transcriptional profile that overlapped glial progenitor cells [21]. Furthermore, RNA velocity analysis showed apical stem cell transcriptional adjacency and velocity vector flow towards the more differentiated tumor cell lineages [21]. Collectively, such findings hint at clear plasticity/fluidity within the tumor. The integration of epitranscriptomics with single cell multi-omics technology could help unveil the yet undiscovered mechanism of how dynamic m⁶A changes play a role in driving plasticity within the tumor niche.

It has been posited that a stem cell may exist at a high or even maximal cellular state of entropy and can readily shift states in the face of perturbation [57]. Our view of the cancer stem cell state in glioblastoma agrees with the theory put forward 20 years ago [58] suggesting that the glioma stem cell is a cellular state or function rather than an entity and this state of maximum cellular entropy is influenced by the constantly adaptable microenvironment of the tumor. In this context, distribution of species would represent heterogeneity, which single cell RNA sequencing adequately captures. Quantum states would equate probability distribution of discrete cell state occupancy bias. In other words, if we looked across a large set of samples and performed, for instance, m⁶A, ATAC-seq, RNA-seq integrated multi-omics single cell analysis, it is possible to generate cell state probability occupancy distribution and ultimately identify discrete, preferred transcriptomic and epitranscriptomic cell state occupancies or quanta states. This will allow to construct an individualized transcriptomic/epitranscriptomic landscape and to find patterns within the seemingly stochastic, chaotic environment that is the tumor.

Can we integrate multiple epigenetic “landscapes” with observed clinical outcomes and use this information on a training predictive model to identify discrete favorable and unfavorable cellular microstates? And ultimately can we target plasticity-based processes to convert the microstate cellular make up of a highly malignant tumor bulk into a less aggressive cellular composition? It is plausible that m⁶A regulatory processes may represent a key target in this endeavor.

6. Conclusion

In this chapter, we introduce GBM in the context of early genetic characterization and suggest that limitations in discrete classification hinted at an inherent cell state fluidity or plasticity. This plasticity may stand as a key function utilized by GSCs and differentiated cancer cells to rapidly and constantly respond to natural and non-natural/therapy induced microenvironmental fluctuations. Epitranscriptomic dynamic changes are explored as a new frontier in epigenetic based adaptation mechanisms. Additionally, single cell multi-omic technology and its yet to occur application to m⁶A can pave the way for improvement in GBM characterization and patient management. Lastly, we theorize that the integration of multi-omic cell technology and m⁶A using massive, high dimensional patient data can aid in the characterization of plasticity through the identification of GBM cell states distribution and quantum state occupancy bias. In the future, such works can be used to develop a Waddington like epigenetic landscape predicting favorable cell state distribution and thus help in the development of plasticity-based therapy to convert glioblastoma into a non-adaptable therapeutic target.

Conflict of interest

“The authors declare no conflict of interest.”

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