Commentary

Insights into the co-evolution of glioblastoma and associated macrophages

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Abstract

Glioblastoma (GBM) is one of the most immunosuppressive and heterogeneous tumors with limited treatment options. Most studies relied on treatment-experienced patient samples to elucidate the origins of tumor heterogeneity, introducing bias into the analysis. The analysis of samples from multifocal GBM patients, in which independent lesions arise from the same progenitor and undergo parallel evolution, enables the study of the natural evolution of GBM while removing the effect of therapy on the emergence of heterogeneity. This enables the identification of critical events in the evolution of GBM and the unbiased study of subtype progression, diversity, and invasive potential. The tumor microenvironment of GBM undergoes significant changes throughout tumor progression. Recent studies have highlighted the switch from an abundance of resident microglia-derived macrophages in earlier stages to the prevalence of blood-derived macrophages in later stages of GBM. There is conclusive evidence that these alterations cannot be viewed in isolation and that the tumor microenvironment co-evolves with tumor cells during cancer progression. Together with an increasingly hypoxic environment, this culminates in highly immunosuppressive conditions, resulting in a feedback loop further reinforcing evolutionary changes in the tumor. A new study now provides a unique look at the natural evolution of GBM, identifies critical events in its development, and has the potential to help improve the diagnosis and therapy of this deadly disease.

INTRODUCTION

Glioblastoma (GBM) is adults' most common and aggressive brain tumor. Despite recent immunotherapy-related success in treating some types of cancer, GBM remains primarily refractory to these approaches, resulting in a continuously low median survival following treatment[1]. The resistance to immunotherapy is primarily accredited to the heavily immunosuppressive tumor microenvironment (TME) commonly found in GBM, in which macrophages can make up to 45% of the total tumor mass[2]. Additionally, the tumor and the associated TME are highly heterogeneous, making it difficult to find viable treatment options[3]. How GBM lesions acquire this heterogeneity and how the interactions between the tumor and the TME influence the evolution of each other throughout tumor development remains challenging to unravel, partly due to limitations in appropriate models. Past efforts have primarily focused on the differences elicited by therapeutic intervention by studying pre- and post-treatment biopsies of GBM patients[4,5]. While these types of study grant insight into later, treatment-induced stages of heterogeneity, they cannot account for the natural progression of single tumor lesions and their interactions with the TME. While relatively rare, multifocal GBM constitutes an ideal model for studying natural tumor evolution. Tumor lesions in multifocal GBM derive from a common precursor undergoing parallel evolution. This offers a unique chance to identify defining events in the developmental progression of GBM that might be clinically exploitable[6]. In a newly published study, Wu *et al.* utilize lesion-specific patient-derived samples of multifocal GBM to uncover critical events in the natural evolution of individual GBM tumors[7].

CURRENT UNDERSTANDING OF GBM EVOLUTION

Mutational and evolutionary forces drive the development of cancer[8]. GBM is believed to arise from a common precursor that finally acquires several rounds of rearrangements, mutations, and transcriptional changes to form invasive tumor lesions[9]. While specific driver mutations in GBM remain controversial, the most commonly mutated genes include *TP53, EGFR, IDH1,* and *PTEN*[10]. Distant recurrent lesions within the same patient have undergone branched evolution by acquiring additional driver mutations and exhibiting a lower retention rate of the initial mutations[5]. GBM lesions progress along a trajectory of three subtypes, each defined by a unique transcriptional signature. Moreover, the TME evolves parallel to the tumor cells, with macrophages accumulating in recurrent lesions in an *NF1* status-dependent manner. Despite an enrichment of T-cells at recurrence, however, resistance to therapy is high[11]. Importantly, whether the genomic evolution, the progression from one transcriptional state to the next, and the changes in the TME also occur naturally in treatment-naïve patients or are a consequence of therapy remained unclear. The mutational status of isocitrate dehydrogenase (IDH) is a marker of favorable prognosis, with *IDH*-wildtype gliomas featuring a more progressed tumor state and shortened patient survival. Most GBM cases are *IDH*-wildtype tumors, and studies including *IDH*-mutant tumors can easily skew results due to their less progressed evolutionary features[12]. To overcome this limitation, Wu *et al.* focused exclusively on *IDH*-wildtype tumors derived from multifocal GBM, completely removing the conflicting effect of *IDH*-mutant tumors on their conclusions[7].

MARKER GENES OF GBM EVOLUTION

Wu *et al.* collected biopsies from two separate lesions of four patients each and subjected them to single-cell RNA sequencing (scRNA-seq) and whole-exome sequencing to create an atlas of over 300,000 single cells[7]. Analysis of chromosome copy numbers and whole-exome sequencing confirmed that both lesions in each patient were derived from a common ancestor. Importantly, further analysis suggested that one lesion

emerged as the offspring of the other lesion in all four patients, confirming the model's validity and enabling the study of events occurring during the natural progression of GBM. To better define the evolutionary history of GBM, the authors established the natural evolution signature (NES), a list of twelve genes most significantly differentially distributed between young and old lesions. All these genes have been previously reported as essential mediators of GBM pathology and are involved in invasiveness, angiogenesis, and immune evasion [Table 1][11]. Tumor cells expressing a high NES (hNES) reached an evolutionary endpoint and are associated with pathways such as apoptosis, angiogenesis, and hypoxia. The NES in a radiation-treated glioma mouse model was significantly higher than in treatment-naïve mice and in recurrent *vs.* primary GBM in human patients, confirming that the NES adequately represents the progression status of GBM. Mechanistically, the NES showed a significant correlation with hypoxia, and GBM cells grown under hypoxic conditions exhibited an increased NES, while this effect was reversed by the knockdown of hypoxia-inducible factor 1 alpha (*HIF1A*). Of note, hypoxia is one of the transcriptional programs reported to be highly variable and a hallmark of GBM heterogeneity[13]. HIF1A activates FOS like 2 (FOSL2), a transcription factor that has been implicated in the development of GBM previously and that can regulate a wide range of target genes, including genes of the NES[14]. As a member of the FOS gene family, it dimerizes with members of the JUN family to form the AP-1 transcription factor. AP-1, in turn, is most prominently activated by MAPK signaling and is active in many different types of cancer[15]. Together, the increased expression of the NES genes represents a more extended evolutionary history and a further progressed tumor state.

SUBTYPE PROGRESSION OF GBM

Identifying unique attributes and establishing universally applicable subtypes remains difficult for GBM and depends strongly on the features utilized for characterization. Historically, GBM has been divided into three to four different subtypes based on bulk RNA sequencing data analysis, including a proneural, classical, and mesenchymal subtype[11,16]. With the technological advances in scRNA sequencing, new subtypes and refined models were introduced. Among the proposed models, the distribution of tumor cells into one of the following four transcriptionally distinct groups has emerged as the most commonly referenced classification: neural-progenitor-like (NPC), oligodendrocyte progenitor-like (OPC), astrocyte-like (AC), and mesenchymal-like (MES) cells. Each of these states correlates with an alteration in the locus of *CDK4, PDGFRA, EGFR,* or *NF1,* and cells transition along an OPC/NPC/AC/MES axis during tumor progression[17,18]. Importantly, Wu *et al.* observed a gradual increase in NES score along the same axis, which, together with survival analysis of TCGA and CGGA GBM datasets, further validated their signature as a prognostic factor[7] [Figure 1]. The mesenchymal subtype is the most detrimental subtype of GBM and exhibits the worst survival rates, emphasizing the potential clinical significance of the NES. However, there was no significant enrichment of the NES in the MES state, pointing to distinct biological functions. GBM cells can also be classified according to the cell state concerning their stem cell properties as stem-like, differentiated-like, or proliferation stem-like glioma cells. While stem-like cells constitute the major cell state in IDH-mutant tumors, IDH-wildtype tumors are enriched for differentiated-like cells[19]. This is remarkable because IDH-wildtype GBM is the most detrimental type of brain cancer, and the older lesions in the multifocal GBM cohort were all enriched for both the stem-like and differentiated-like states, while the younger lesions were enriched for the two stem-like cell states only. This further underlines the accuracy of the NES regarding prognosis and tumor state.

CO-EVOLUTION OF THE TME

The primary cell types other than tumor cells across all lesions in multifocal GBM were myeloid cells, followed by oligodendrocytes, fibroblasts, T-cells, and endothelial cells. Macrophages are a highly heterogeneous group of cells that commonly accumulate in tumors, and their characterization into

Table 1. The NES genes and their proposed function in GBM

| ID	Type	Function in GBM
S100A10	Receptor	Hypoxia, immune response, & invasion[39-41]
FOSL2	Transcription factor	Angiogenesis & plasticity[14,42]
SPP1	Ligand	Immune response[43]
CAV1	Cell surface protein	Tumor progression & signaling[44,45]
ANXA1	Ligand	Immune response
VIM	Structural protein	Invasion[46]
CD44	Receptor	Invasion & angiogenesis[47]
SERPINH1	Protease inhibitor	Immune response[48]
LGALS3	Ligand	Immune response & resistance to therapy[49,50]
CEBPB	Transcription factor	Proliferation & resistance to therapy[51,52]
ATF5	Transcription factor	Proliferation[53]
LGALS1	Ligand	Immune response & resistance to therapy[54,55]
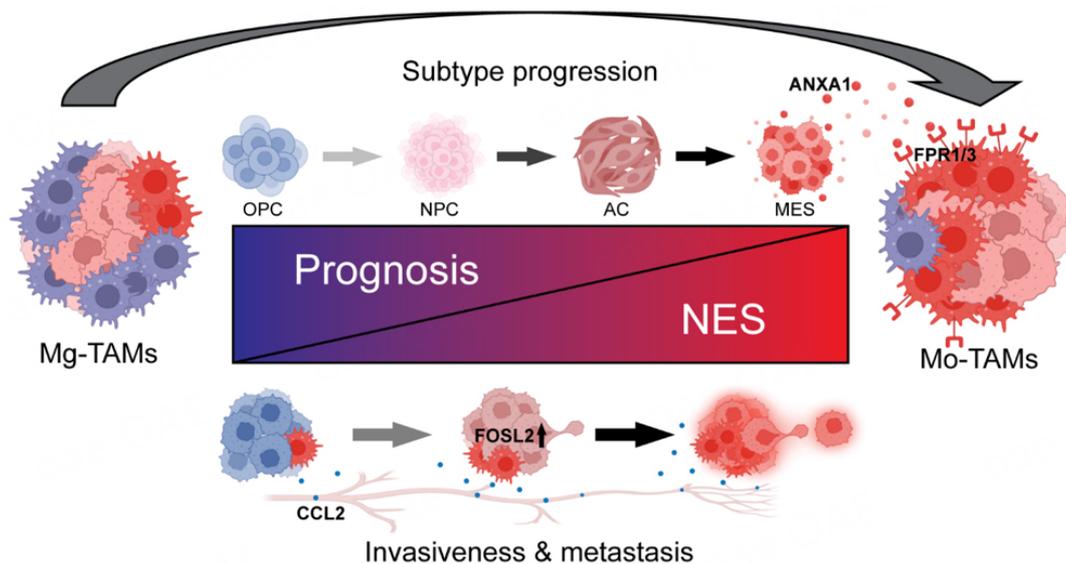
Figure 1. The co-evolution of glioblastoma. An increase in the natural evolution signature indicates poor outcome and is emphasized by the progression of cellular subtypes from OPC (oligodendrocyte progenitor-like) to NPC (neural progenitor-like), AC (astrocyte-like), and finally MES (mesenchymal-like). Activation of HIF1A/FOSL2 signaling in tumor cells leads to the secretion of ANXA1, which binds to FPR1/3 on monocyte-derived macrophages (Mo-TAMs), recruiting them to the tumor where they gradually replace the resident microglia-derived macrophages (Mg-TAMs), ultimately resulting in an increasingly immunosuppressive tumor microenvironment. Activated Mo-TAMs secrete CCL2, which, together with the activation of FOSL2 in tumor cells, further increases the invasive potential of GBM cells.

functional subsets remains challenging[20]. Previous studies have utilized scRNA-seq data to establish gene signatures to reliably characterize the origin of the tumor-associated macrophages (TAMs) in GBM as either monocyte-derived brain macrophages (Mo-TAMs) or microglia-derived macrophages (Mg-TAMs). Interestingly, Mo-TAMs constitute the main myeloid population in recurrent GBM, while Mg-TAMs are enriched in newly diagnosed GBM[21]. In agreement with this, the older lesions in the multifocal model harbored an increased number of Mo-TAMs, while the younger lesions showed a high number of Mg-TAMs [Figure 1]. Accordingly, a previously established bone marrow-derived macrophage (BMDM) signature also correlated with the NES[22]. Based on the Ivy GBM Atlas Project, GBM is divided into five different regions: the cellular tumor region, infiltrating tumor (IT) region, leading-edge (LE) region,

microvascular proliferation (MP) region, and pseudopalisading cell (PC) region[3]. According to this characterization, the spatial distribution of Mo-TAMs and hNES tumor cells also correlated, with both cell types showing similar enrichment in the MP and PC regions, while Mg-TAMs were primarily located in the LE regions. The PC region is located at the center of the tumor, is associated with necrosis, and is considered an older part of the lesion. Together, this confirms that the progression of the tumor cell NES is not only associated with tumor progression but also tied to the shift in macrophages from Mg-TAMs to Mo-TAMs. Moreover, macrophages can induce the transition of tumor cells to the MES state, tying together tumor evolution with subtype progression and changes in the TME[23]. Further analysis confirmed that the communication between tumor cells, myeloid cells, and T-cells was the dominant interaction interface among overall cell communication, with macrophages acting as intermediaries between tumor and T-cells. The interaction between macrophages and hNES tumor cells was more pronounced in the older lesions, implying a functional relationship in shaping the TME. Interestingly, the only other cell type differentially distributed between younger and older lesions was oligodendrocytes, whose distribution was inversely correlated to macrophages and which accumulated in the younger lesions in the same three out of the four patients.

MECHANISTIC INSIGHT INTO TUMOR CELL-MACROPHAGE INTERACTIONS

An ANXA1-FPR1/3 axis dominated the interaction between macrophages and tumor cells in all four patients. Annexin-1 (ANXA1) seemingly acts as an oncogene in GBM, correlates with poor prognosis, and was suggested as a prognostic marker. Moreover, the proportion of infiltrating immune cells showed significant differences in ANXA1 high and low GBM patients, further suggesting an essential role in shaping the TME[24,25]. The main interaction partners of ANXA1 are the formylated peptide receptors 1-3 (FPR1/2/3), which are commonly found on immune cells and whose activation is associated with reduced leukocyte migration and adhesion and, thus, reduced inflammation[26]. A whole-body knockout of FPR1 in mice corroborated the reduction in inflammation and, more specifically, a significant decrease of M2 macrophages[27]. M2 macrophages are considered immunosuppressive and a significant hurdle in the immunotherapeutic treatment of GBM[28]. In the older lesion of the multifocal tumor patients, ANXA1 mainly interacted with FPR1 and 3. FPR3 being highly expressed on Mo-TAMs but not Mg-TAMs hints at a function in the recruitment of BMDMs rather than the activation of resident microglia. Further *in vitro* and *in vivo* experiments confirmed the role of ANXA1 in the recruitment of monocytes and their differentiation into an M2-like state, ultimately leading to reduced T-cell activation and proliferation and poor survival in mice. ANXA1 appears to be induced by FOSL2, tying together the increasingly hypoxic environment of GBM with the reshaping of the TME via a HIF1A-FOSL2-ANXA1-FPR1/3 axis.

DEVELOPMENT OF INVASIVE POTENTIAL

This study is consistent with previous observations that Mo-TAMs accumulate in recurrent GBM relative to their matched primary tumors, indicating an active role of BMDMs in shaping tumor evolution[21]. Indeed, co-culture experiments, GBM stem cell cultures, and RNA sequencing showed that Mo-TAMs could induce the NES in tumor cells. Epithelial-to-mesenchymal transition (EMT) is a defining step for tumor cells to acquire these invasive capabilities and, thus, tumor progression[29]. The observed increase in the NES in tumor cells interacting with Mo-TAMs correlated with a concomitant increase in EMT, following an increase in *FOSL2* expression. FOSL2, and in extension AP-1, are part of a transcriptional network crucial in promoting EMT[14,30]. Metastatic gene signatures and expression data from diffuse glioma cells with increased migratory abilities also correlated with a high NES score, confirming that the NES represents an endpoint in GBM tumor evolution and a prognostic marker. The positive effect of macrophages on the migration of tumor cells is well known; however, the mechanisms underlying this interaction are still not fully understood. While it seems to depend mainly on cytokine signaling, the exact types of cytokines and

the context favoring their release remain dark[31]. Mo-TAMs in the older multifocal lesions expressed high levels of CCL2, which acted as a potent inducer of tumor cell migration, providing a further explanation for tumor progression and the increasingly invasive potential in this model GBM evolution [Figure 1].

FUTURE DIRECTION AND OPEN QUESTIONS

While it is apparent that the NES and the reshaping of the TME share a causative relationship, several factors are still not fully disclosed. First, the exact timeline of NES progression, tumor cell evolution, and TME remodeling awaits to be uncovered. Even though the NES progresses along with the GBM subtypes, the clinical importance of the NES also remains questionable since the NES showed no significant enrichment in the MES state. Second, it is still unclear which critical signaling events drive this transition. Furthermore, since the authors focused on the role of a specific ligand, the impact of distinct cell surface receptor expression on target cells was not assessed in this study. Since macrophages grown *in vitro* do not resemble the myriad of cell states found *in vivo*, information about immunosuppressive and inflammatory cell states, ligand and receptor expression, and general cell plasticity gained from *in vitro* studies must be carefully evaluated. Lastly, this study focused on the interaction of tumor cells and macrophages. The TME, however, is a collection of various cell types and extracellular components whose contribution to tumor evolution is a matter of speculation. Apart from T-cells, the role of other immune and non-immune cells, such as fibroblasts, pericytes, and endothelial cells (ECs), remains unknown. The differentiation of glioma stem cells into glioma-derived ECs, for example, is crucial to support the vascularization and invasiveness of GBM, and it will be interesting to see how the NES is connected to events such as these[32,33].

As with any type of cancer, timely detection is one of the most deciding factors in survival. It seems clear that the NES is a reliable prognostic marker in GBM. If, however, this can be exploited to improve diagnostic methods remains to be seen. An ideal diagnostic marker would be a secreted factor produced in the early stages of cancer or even pre-cancerous lesions, which is detectable in the serum. Interestingly, serum CCL2/3 has previously been proposed as a prognostic biomarker in a different type of cancer[34]. Besides serving as a diagnostic marker, CCL2 might also represent a potential target in combinatorial therapies. Indeed, targeting macrophages in immunotherapy has garnered newfound interest in recent years, and several compounds are currently undergoing clinical trials[35].

While GBM exhibits, one of the highest numbers of macrophages, and its TME is among the most immunosuppressive, several other tumor types also boost many immunosuppressive macrophages and are largely refractory to immunotherapy[36]. This begs the question whether the NES established in this work is more broadly applicable or whether each tumor type features a unique set of genes throughout its evolution. Proteins, such as FOSL2, HIF1A, and CCL2, appear to be a more common feature of cancer development, which might hint at some conserved signaling axes in tumor evolution between different organs.

Lastly, this study found that macrophages were not the only cell type differentially distributed among the older and younger lesion. Contrary to Mo-TAMs, oligodendrocytes were increasingly lost in older lesions. Under normal conditions, oligodendrocytes are responsible for the myelination that provides insulation for the axons in the brain[37]. While their role in GBM remains incompletely understood, they seem to serve a pro-tumorigenic function, as they can promote neovascularization and help disrupt the blood-brain barrier[38]. In light of this, their dwindling in older lesions is unexpected and warrants further exploration.

CONCLUSION

This study by Wu and colleagues provides a glimpse at the natural evolution of GBM at single-cell resolution. The authors uncover a HIF1A-FOSL2-ANXA1-FPR1/3 signaling axis that is ultimately tied to

the recruitment and polarization of macrophages and an increase in tumor cell invasiveness [Figure 1]. This study represents only the beginning of unraveling the interplay between GBM stem cells, differentiated tumor cells, tumor-infiltrating, resident macrophages, other parts of the TME, an increasingly hypoxic environment, and the consequences for diagnosis and therapy.

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