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Treating glioblastoma often makes a MES

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Glioblastoma (GBM) brain tumour cells exhibit pronounced phenotypic plasticity, but exactly how this enables GBMs to inevitably resist standard treatment is not known. A new study uses multi-level molecular profiling of pre- and post-treatment human GBMs to shed light on treatment response with single cell and spatial resolution.

The 90% of glioblastoma (GBM) brain tumours that do not harbour mutations in isocitrate dehydrogenase genes (IDHwt GBM) are the deadliest. Surgical debulking followed by chemoradiotherapy with alkylating agent Temozolomide constitutes standard of care but only increases survival, on average, from 12 to 15 months. The urgent clinical need to identify effective treatments, for GBM led to it being one of the first tumor types to have extensive genomic characterization. However, genotoxic treatments that target the most common somatic mutations in GBM have repeatedly failed in clinical trials. Global efforts to characterise longitudinal GBM samples revealed a lack of evolutionary bottleneck imposed by standard treatment[1,2]. This suggests that, at diagnosis, the cancer genome is no longer the main driver of malignant progression in GBM. Since then, transcriptionally defined GBM cell types have been identified, with evidence of widespread inter-conversion and cellular plasticity that was not previously realised[3-6]. Proportional changes in cell types, characterised using longitudinal bulk tumour data, intimates that treatment shapes the GBM cellular landscape in non-stochastic ways[7]. In this issue of Nature Cancer, Wang et al[8] have reaped the rewards of decades of brain tumour tissue banking to provide a single cell atlas of pre- and post-treatment IDHwt GBM tumours. They have performed single nucleus RNAseq on frozen archival material, alongside scATACseq, spatial proteomics and spatial transcriptomics on subsets of their cohort. They have identified recurring therapy-driven shifts in neoplastic cell populations, in agreement with bulk tissue studies, but then been able to expand on this to identify candidate mechanisms underpinning the shift. Such mechanisms may hold the key to therapeutically targeting GBM adaption to treatment.

Findings from several single cell RNAseq analyses of patient GBM tumours[3-6]. have dovetailed into the realisation that a variety of neoplastic cell types, defined by transcriptional programmes, are shared across patients. The number and nomenclature of cell types has varied, but there is significant overlap in the classifications and a consistent message of plasticity and interconversion. The Diaz group, whose most recent work is the focus of this highlight[8], previously identified a single axis of variation with proneural-like (PN) stem cells at one end and mesenchymal-like (MES) stem cells at the other[5]. Neftel et al. identified four GBM cell subgroups denoted OPC (oligodendrocyte progenitor-like cells), NPC (neural progenitor-like cells), APC (astrocyte-like cells) and MES (mesenchymal-like), all able to proliferate[3]. Inspecting Neftel cell types in relation to single cell methylation profiles indicated that NPC & OPC together appear to mirror Diaz's PN subtype, while APC and MES subgroups together mirror Diaz's MES subtype[9]. In all cases, transcriptionally defined GBM cell types span genomic subclones and, whilst there is somatically encoded predisposition to a given neoplastic cell type, the delineation is driven epigenetically, as it is for phenotypically distinct normal brain cell subpopulations. This produced a paradigm shift: the notion that the genome is not the main driving force underpinning malignant progression of GBMs once these tumors are established, and thus may not be the ideal source of much needed targets for effective therapy. Thus, an alternative way forward warrants understanding the phenotypic plasticity of GBM cancer cell populations and mapping out their trajectories to learn how to negate their ability to adapt and survive.

In this latest effort[8], Wang et al performed single nucleus RNAseq on 36 pairs of primary and matched recurrent IDHwt GBM tumours from patients that received standard treatment. They also profiled 4 unmatched primary and 5 unmatched recurrent samples. Extend prior findings[5], they show that the largest source of transcriptomic variation is along the PN to MES axis in the treated recurrent samples as well as in untreated primaries. Classifying cells into either subtype revealed a significant increase in the proportion of MES cells at recurrence, per patient on average. A therapy-related switch to mesenchymal-like tumours has been repeatedly observed in bulk genomics studies of glioma, often associated with changes in the tumour microenvironment (TME)[10]. The unprecedented single-cell resolution achieved by Wang et al.[8] shows that there are more MES cells at recurrence but also that they become more strongly polarised toward the MES classification. Furthermore, uniquely within

tumours that underwent a MES shift, there was a significant increase in cycling cells. This MES shift could be due to several phenomena: MES cells may be more inherently resistant to treatment, so become enriched; non-MES cells may convert to MES cells in response to treatment; MES cells may proliferate more, and/or more MES cells are produced during cell division, than other neoplastic cell types. Inspecting MES cells from recurrent GBMs, Wang et al.[8] identified that the largest source of variation was between quiescent cells expressing TGF beta pathway genes and proliferating MES cells with upregulated DNA damage response programmes. Alongside results from RNA velocity, this suggests that treatment prompts quiescent MES cells to begin proliferating to produce an overall increase in MES cells in recurrent tumours.

Wang et al.[8] then explored mechanisms underpinning a MES switch. From their snRNAseq data, they inferred somatic losses of Chr6q or Chr14q, and gain of Chr19 or Chr20 that were significantly more prevalent in MES than PN cells, suggesting that MES predisposition genes reside in these regions. Four of the same GBM sample pairs underwent single cell chromatin accessibility profiling using ATACseq, yielding two clusters of cells. The transcription factor bindings sites that were uniquely accessible in one cluster clearly aligned with the PN classification e.g. OLIG2 and NEUROG1. The other cluster had significant availability of binding sites associated with the AP1 transcription factor complex, which has previously been implicated in regulating the MES phenotype[6,10,11]. Herein, Wang et al.[8] integrated their RNAseq and ATACseq data to infer an AP1 regulome in GBM, highlighting downstream effectors that may drive mesenchymal transformation at the single cell level. I*n vitro* and *in vivo* experiments showed that: 1) AP1 components, and some target genes, are induced by ionising radiation; and 2) drugs that inhibit candidate AP1-regulated genes are synergistic with non-surgical components of standard GBM treatment, though this was somewhat cell-line and drug dependant. Together these data show that the MES cell transcriptional programme is induced by treatment, and prompted the authors to suggest that MES drivers constitute therapeutic targets. It should be noted however, that whilst there was an increase in MES cells on average per patient in recurrent vs primary GBM, some cells also polarized in the opposite direction, towards the PN classification. Interestingly, it appeared that any primary tumour with a low proportion of either PN or MES cells recurs with an increased proportion of that cell type, though the trend was stronger for primary tumours with a low proportion of MES cells. Chromatin accessibility data showed a significant increase in relative size of MES clusters at recurrence but not to the point of dominating the tumour mass (a 40% to 49% increase). Hence whilst treatment alters the neoplastic cell landscape, often toward a mesenchymal programme, further research is needed to understand reprogramming in alternative directions at both the tissue and cellular level. Emergent approaches that enable longitudinal tracking of whole transcriptomes in individual cells will be invaluable to understand the true plasticity of GBM cancer cells over time in response to treatment[12].

Wang et al.[8] also investigated how treatment changed the cellular make-up of the TME. They found no change in proportion of innate immune cells overall but further classification revealed a significant increase in bone-marrow derived monocytes, and decrease in resident microglia, in recurrent samples. Most innate immune cells displayed a nonpolarized “M0” state at both primary and recurrence, but there was a significant increase in the number of activated (M1/M2) cells through treatment. T-cells were infrequent, 1% on average, and most commonly in an exhausted state; more so at recurrence than in the primary tumour. However, 16% of recurrent GBMs were found to have 2-20 fold more T-cells than average. These cases had improved survival and, at recurrence only, the amount T-cells correlated with the tumour mutational burden. Spatial proteomics on 3 pairs of “T-cell outlier” samples revealed tertiary lymphatic structures in recurrent GBMs that were also enriched for B-cells and cells of monocytic lineage. This suggests that in some cases, in contrast to what has been reported by bulk genomics studies that are unable to delineate the cellular make up of tumours in such resolution[13], GBMs with higher mutational burden could be more immunogenic, in part owing to treatment driven effects.

Recent excitement within the GBM field has stemmed from the identification of extensive interactions of GBM cells with each other and with normal brain cells, through tubule networks and electrical synapses[14,15]. Wang et al.[8] inferred cellular crosstalk from their snRNAseq data, finding strikingly similar communication patterns in primary and recurrent GBMs, but with more potential communication routes associated with MES tumor cells, and seemingly more emanating from astrocytic cells, in the recurrence. Spatial transcriptomics validation showed that many predicted receptor:ligand interactions became more prevalent (4-10x) upon moving from the core to the infiltrative edge of the tumour. In particular IGF and WNT pathways were enriched in this manner, with ligand-expressing cancer cells and receptor-expressing normal glia. Conversely, PTN:PTPRZ1 expression patterns indicated

neoplastic cell autocrine signalling that was significantly more prevalent in central, dense tumour regions. To inspect the relevance of these findings, paracrine signals were added to *in vitro* models, which altered cell proliferation and, in some cases, treatment response with variable synergistic effect.

In summary, this study provides a compendium of single cell data from longitudinal IDHwt GBM samples, enabling detailed inspection of molecular trajectories of GBM cancer cells exposed to standard treatment. Integration of spatiotemporal datasets at different molecular levels enables the authors to move from describing cell type shifts to elucidating the mechanisms underpinning them, which represent strong potential therapeutic targets. However, integration of independent datasets at snapshots in time come with inherent limitations. As technology advances the ability to produce multi-omics data from the same cell and perform longitudinal tracking of viable cells, will further increase our understanding tumour adaption mechanisms. This study, which came to fruition following years of high-quality tissue-banking, highlights the need for such foundation research resources, alongside rapidly advancing technologies. The validity of the findings from this phenomenal single cell atlas are mostly borne out by several *in vitro* and *in vivo* experiments probing certain candidate mechanisms. However, it also highlights that lab-based inspection of GBM patient-based findings often leads to confounding results that are dependent on the choice of model. This further emphasises the need for a range of well characterised experimental systems for GBM research to maximally leverage resources from clinical datasets such as these, and to help translate findings from bedside to bench and back again.

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