CANCER

Now you see me; now you don't

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Multiomics on serial glioblastoma biopsies can enable differentiation of pseudoprogression from true tumor progression (see Ling *et al.*).

INTRODUCTION

Seeing is believing, and seeing tumor growth is essential to both diagnosing and treating incurable glioblastomas (GBMs). Until now, seeing tumor growth has been limited to the use of magnetic resonance imaging (MRI). However, in a tour de force work, Ling *et al.* (*1*) demonstrate that serial biopsies and multiomics effectively see what remains invisible to the MRI. The results are groundbreaking and may rewrite how glioblastoma is diagnosed, how its progression is followed, and how it gets treated.

Ling *et al.* conducted a feasibility study of serial multiomic analyses of recurrent GBM biopsies from two patients treated with the oncolytic immunotherapeutic agent CAN-3110 (*2*, *3*). The study, although limited to two patients, demonstrates the value of longitudinal tissue sampling and multiomic profiling to understand GBM's complex evolution, especially when standard clinical assessments such as MRI can be limited or even misleading. Despite MRI-diagnosed tumor progression in these participants, multiomic analyses revealed therapeutic effects, including reshaping of the tumor microenvironment (TME), expansion of CAN-3110–reactive and other T cell clonotypes, and induced expression of human leukocyte antigen (HLA)–presented immunopeptides. Both patients showed positive clinical outcomes (pathologic response or stable disease), challenging reliance on conventional imaging for assessing immunotherapy efficacy in GBM.

EVALUATING GBM TREATMENTS BY MRI

GBM remains an incurable and aggressive brain cancer with a very poor prognosis; the 2-year survival rate stands currently at 5% (*4*). A critical challenge in developing effective GBM therapies is our capacity to assess the effects of the various new treatments and to separate beneficial therapeutic responses from disease progression. As said poetically by Pink Floyd, "So, so you think you can tell, Heaven from Hell, blue skies from pain…." Currently, we rely on MRIs to assess treatment outcomes and disease progression, but this approach has limitations. MRIs are evaluated mainly on the basis of the presence and distribution of gadolinium enhancement [i.e., T2/fluid-attenuated inversion recovery (FLAIR) hyperintensity]. The location of the anatomical lesion is detected quite accurately by MRI; however, the exact pathological anatomy underlying the images is less clear. Although MRI is very good at assessing tumor responses to chemo- and radiotherapy and distinguishing whether the tumor has grown, shrunk, or remained the original size, MRI cannot inform about whether an experimental therapeutic agent has reached the tumor mass or whether the agent engaged its potential target. More crucially, MRI is unable to differentiate inflammatory responses from actual tumor progression (*5*). Current MRI imaging approaches cannot discern whether growth in a mass is due to actual tumor growth or to inflammatory changes in response to treatments, a phenomenon termed pseudoprogression. This is challenging when trying to interpret changes to the tumor, especially when such inflammatory changes are intended to be caused by treatments such as immunotherapies. Much clinical detective work has led to the concept of pseudoprogression (*6*), but currently the only way to determine the difference between pseudoprogression and true tumor progression is pathological evidence in favor of either diagnosis. This is rarely done, and so pseudoprogression is mostly diagnosed a posteriori, when follow-up evidence fails to detect continued tumor growth. Complete trust in MRI to determine disease stage, especially in the case of novel immunotherapies, is thus limited (*7*).

Window-of-opportunity trials and phase 0 trials have thus been proposed to overcome some of the shortcomings indicated above (*8*). These can provide accurate values of amount of drug present in a human tumor in vivo yet are limited to a single time point, precluding a deeper understanding of the kinetics and evolution of the effects of treatments. How would it be possible to overcome these limitations?

SERIAL BIOPSIES OFFER A WINDOW INTO TRUE TUMOR EVOLUTION

Ling *et al.* used serial planned tumor biopsies to improve diagnostic power, combined with repeated intratumoral injections of the oncolytic immunotherapeutic virus CAN-3110 to improve therapeutic efficacy (Fig. 1). Several recent technological advances enable this complex approach: (i) the ability to perform sophisticated molecular analysis on small tissue samples; (ii) the capability of state-of-the-art bioinformatics to perform parallel analysis of cellular, molecular, and immunological phenotypes simultaneously at the DNA, RNA, protein, and metabolite levels; and (iii) improvements in the safety and precision of image-guided brain tumor biopsies.

For the study design, after a pretreatment baseline biopsy of recurrent tumors, patients were subjected to biopsies up to five times in a span of 4 months. From the first two patients (of an eventual total of 12) reported in this manuscript (*1*), 97 serial multisector biopsies were collected and analyzed by multiomics, including cyclic immunofluorescence, single-cell RNA sequencing, proteomics, phosphoproteomics, immunopeptidomics, and tumor T cell clonotype characterization, as well as routine histopathology. A dose of CAN-3110, an oncolytic immunotherapeutic agent derived from herpes simplex virus 1 (HSV-1) (*2*), was also injected at each biopsy time point.

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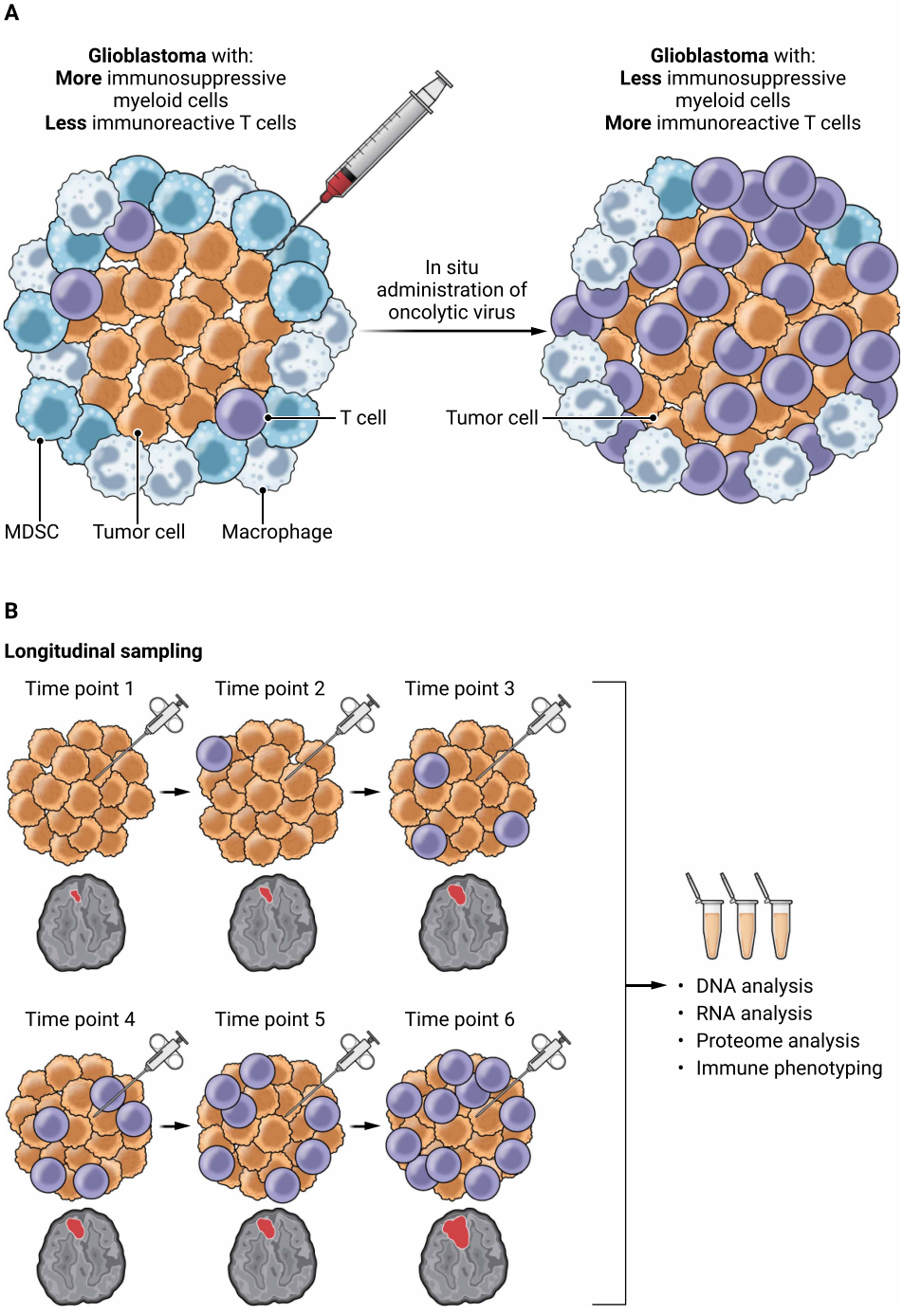

Fig. 1. Separating progression from pseudoprogression. (**A**) Ling *et al.* (*1*) demonstrated that, after treatment with oncolytic HSV-1, the GBM microenvironment evolves from cold and immunosuppressive (left) to hot and inflamed (right), with a reduced proportion of immunosuppressive myeloid cells and an increased proportion of immunoreactive T cells. (**B**) Evolution of the serial administration of oncolytic HSV-1 coupled to serial biopsies used for molecular, proteomic, and immune analysis over long time periods of disease evolution. The MRI schematics underneath the tumors indicate the complexities of tumor imaging, which fails to predict exactly the status of immunotherapy treatment. The serial biopsies, however, detect a more faithful picture of tumor status, can separate tumor progression from pseudoprogression, and allow an in-depth understanding of tumor evolution and its response to immunotherapies.

The procedures were well tolerated, and there were no dose-limiting toxicities. However, both patients' MRIs showed progressively increased contrast enhancement consistent with, according to standard neuro-oncology RANO (response assessment in neuro-oncology) criteria (*4*), tumor progression. This led to one patient withdrawing from the trial.

Unexpectedly, and in contrast with the MRI findings, the multiomic and histopathologic analyses showed evidence of therapeutic effects and immune activation in both

patients. The neuropathology of serial biopsies showed increases in inflammatory cells, plasma cells, lymphocytes, and mononuclear cells, suggestive of an active immune response accompanied by decreased tumor presence compared with baseline data in both patients. The areas of remaining tumor cells were inversely correlated with the nearest prior site of injection of CAN-3110, suggesting the existence of a halo of therapeutic effect surrounding each injection site. These data demonstrate that the treatment caused a reshaping of the TME vis-à-vis the tumor content remaining, including an appreciable shift in tumor subtype from astrocyte (AC)–like to mesenchymal (MES)–like in response to treatment.

Similarly, major shifts in the TME were detected by cyclic immunofluorescence. Increased immune activation was observed in a number of immune measurements: $CD45^+$ leukocytes, $CD163^+$ macrophages, and $CD8^+$ T cells all increased after therapy, with lower tumor amounts and higher immune molecular signatures in a halo surrounding the more recent sites of CAN-3110 injection. In addition, there were longitudinal changes in HLA up-regulation, novel immunopeptide expression, effector T cell expansion, emergence of new T cell clonotypes, and increases in the frequencies of HSV/CAN-3110–reactive T cells. Encouragingly, these changes were seen in both patients, highlighting the disconnect between MRI data and multiomics data. In short, the TME shifted upon treatment with CAN-3110 from tumor-rich to immune-rich, usually referred to as the conversion from a "cold" to a "hot" TME (*9*).

Salient features of this massive rearrangement of the TME included an elevated abundance of up-regulated immunopeptides specific to cancer and up-regulation of signaling pathways related to receptor tyrosine kinase signaling, interferon responses, and apoptosis. Of particular importance was the increase in effector $CD4^+$ and $CD8^+$ T cells, indicating that the treatment had induced an immune response that was able to navigate to the tumor area to destroy tumor tissue. There was also a time-dependent increase in HSV/CAN-3110–reactive T cells, indicating that the immune response was able to recognize both tumor and virus antigens. Preexisting anti–HSV-1 immunity may play a role in regulating the antiviral arm of the immune response (*2*). Eventually, it will be important to determine the particular killing specificities of antitumor and antiviral T cells.

Clinically, the two treated patients achieved either a pathological response or a stable clinical disease; one patient died 12 months after the initiation of study therapy, and the other remained clinically stable for 18 months, at which time they developed tumor progression and initiated salvage therapy. Notably, all clinical and investigational responses were observed despite MRIs indicating disease progression. These results show that complex multiomics is able to distinguish whether MRIs indicating progression are the result of true tumor progression or actually a consequence of treatment.

MOVING FORWARD

The conclusions and implications of this work are wide ranging. Even with the limitations of reporting results for only 2 of a total of 12 patients, these findings could represent a potential paradigm shift in how GBM treatments are followed over time and how treatment is administered. Among the most important findings are (i) the demonstration of the safety and feasibility of repeated longitudinal tumor biopsies over many months, (ii) the superiority of multiomics applied serially to uncover therapeutic effects that cannot be detected by routine MRI or standard clinical analysis, and (iii) the evidence of elicited antiviral and antitumor immunity at sites of treatment delivery. These findings challenge the reliance on MRI for detection of tumor progression, treatment delivery, and overall clinical evaluation and support adoption of a paradigm using serial biopsies and multiomics to select, identify, and prioritize novel GBM treatments.

Are these data enough to influence clinical practice? If so, additional hurdles must be overcome. One practical challenge to overcome for some patients is whether insurance providers will pay for additional surgeries and treatment. If the data support better patient outcomes, then it is likely that insurance companies will be convinced to absorb the extra costs. Likewise, the implementation of the technological complexity may only be feasible at the largest research centers able to complete the kind of trial and treatment described herein. Equally challenging will be the decision on how to determine the effectiveness and toxicity of immunotherapies for the treatment of brain tumors. For CAN-3110, a total of six patients [including the two reported in Ling *et al.* (*1*)] will receive a total dose of 1×10^8 plaque-forming units of CAN-3110 per total injection, and six will receive a 10 times higher dose. Thus, toxicities not seen at the lower dose could be detected at the higher dose of virus. Nevertheless, the Bayesian optimal interval design (BOIN) is likely to ensure the determination of an effective nontoxic dose (*10*).

CONCLUSION

The study by Ling *et al.* (*1*) has the opportunity to reshape care for patients with GBM, both from a monitoring perspective and in terms of immunotherapy design. It is likely that, as a minimum, and if the next cohort of patients display similar responses, the proposed methods of this manuscript will cause a realignment in our treatment of GBMs, with less reliance on MRI as a singular readout of therapy success. At a maximum, it may initiate a veritable revolution in both the assessment and treatment of GBMs, including our understanding and evaluation of virotherapies and immunotherapies such as CAN-3110.

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