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## Greater than the sum of its parts: Single nucleus sequencing identifies convergent evolution of independent *EGFR* mutants in GBM

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### Summary

Single cell sequencing approaches are needed to characterize the genomic diversity of complex tumors, shedding light on their evolutionary paths and potentially suggesting more effective therapies. In this issue of *Cancer Discovery*, Francis et al., develop a novel integrative approach to identify distinct tumor subpopulations based on joint detection of clonal and sub-clonal events from bulk tumor and single nucleus whole genome sequencing, allowing them to infer a subclonal architecture. Surprisingly, the authors identify convergent evolution of multiple, mutually exclusive, independent *EGFR* gain of function variants in a single tumor. This study demonstrates the value of integrative single cell genomics and highlights the biological primacy of *EGFR* as an actionable target in GBM.

### Text

Tumors are greater than the sum of their parts. Cancers are not merely a collection of cells, but rather a complex ecosystem composed of cells that mutate and adapt; interact and compete. The overall behavior of the tumor is shaped by the balance of cooperation and competition between individual cells that can vary in genetic, phenotypic and functional properties (1,2). Tumors evolve in response to treatments, including targeted therapies, through classical Darwinian selection and by cellular adaptations at the DNA, RNA or protein level. Determining the landscape of mutations shared between tumor cells and identifying mutations that arise to provide selective advantage to subclones could provide critical insight into the mechanisms of tumorigenesis, progression and evolution. Further, dissecting the mutational hierarchy of a tumor may identify the pre-existing seeds of drug resistance, thus becoming a key component of more effective, personalized cancer treatment.

Glioblastoma (GBM), the most common malignant primary brain cancer of adults, is one of the most molecularly characterized forms of cancer (3,4). Mutations in coding regions that

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occur at greater than 5% above background are likely to have already been detected (4). A compelling picture from these studies has emerged pointing directly at EGFR as a target. Fifty-seven percent of GBMs show evidence of mutation, rearrangement, alternative splicing or focal amplification of *EGFR* (3). A number of independent, structural rearrangements, including *EGFRvIII* and extracellular domain point mutations that confer gain of function and enhancement of tumorigenicity have been identified (3). Despite the strong evidence, skepticism regarding the importance of EGFR in GBM still remains, largely due to the failure of EGFR tyrosine kinase inhibitors (TKIs) in the clinic. Resistance to EGFR TKIs may be attributed to a number of factors, including: 1) sub-therapeutic dosing of EGFR TKIs (5,6); 2) bypass signaling pathways including through other receptor tyrosine kinases (7) and 3) reversible loss of extrachromosomal *EGFRvIII* DNA (8), all of which suggest the need for developing better EGFR-targeted treatments. Another, less well-understood aspect, is the role of intratumoral heterogeneity of *EGFR* mutants in GBM, and its impact on resistance.

GBM is one of the most heterogeneous of all cancers. Individual tumor cells vary in shape, size, and expression levels of signaling proteins including *EGFRvIII* (7). Fluorescent in situ hybridization studies also show that other oncogenic receptor tyrosine kinases (RTKs), including *PDGFRa* and *MET* can be co-amplified with *EGFR*, either in the same or different tumor cells (9,10). Genomic assessment of bulk tumor tissue cannot resolve the landscape of driver mutations at the single cell level. At present, it is unclear whether multiple *EGFR* mutations occur in individual cells, and whether they co-exist with amplification of wild type *EGFR* and/or other RTKs. Single cell sequencing techniques are crucial for answering these questions.

In this issue of *Cancer Discovery*, Matthew Meyerson's and Keith Ligon's laboratories present a novel single cell sequencing approach to identify unique, non-overlapping sub-clonal alterations from archived clinical samples, shedding new light on the clonal diversity of *EGFR* alterations within GBM. Francis *et al.* (11) examined bulk RNA sequencing analysis of 76 TCGA GBM samples with validated focal *EGFR* amplification and detected focal *EGFR* amplification coexisting with at least one *EGFR* variant, including structural alterations and/or missense extracellular domain mutations, in 71% of the samples. Deeper joint DNA and RNA sequencing analysis of an additional 25 cases revealed remarkable diversity of *EGFR* alterations. Transcripts encoding the same *EGFR* mutant were shown to possibly arise from distinct DNA templates within the tumor and some tumors contained multiple *EGFR* structural variants and missense mutations. In one particularly illuminating example, the possibility of up to 32 different possible clonal combinations based on five distinct *EGFR* genomic lesions was raised. It is possible that other mutational targets also have considerable diversity, suggesting that the overall clonal diversity could be much larger.

To begin to dissect the clonal and subclonal diversity of *EGFR*, Francis and colleagues developed an integrative method to study two GBM samples with focal *EGFR* amplification in depth (11). This novel integrative approach, summarized in Fig. 1A, coupled single nucleus sequencing with bulk tumor analysis, enabling the authors to estimate the clonality of somatic mutations and to infer a subclonal architecture of the tumors.

Each of the two tumors revealed a strikingly different path to oncogenic EGFR signaling (Fig. 1B). All of the tumor nuclei from tumor 1 contained a mixture of both wild type *EGFR* and *EGFRvIII*. *EGFRvIII* arises from an in-frame genomic deletion of exons 2–7 (7). Further, the common amplification boundaries and the *EGFRvIII* deletion breakpoints are shared among the tumor nuclei, suggesting that the amplification of *EGFR* preceded the emergence of the *EGFRvIII* mutant. The levels of *EGFRvIII* DNA varied greatly between cells, consistent with the possibility that extrachromosomal *EGFRvIII* DNA levels could be responsive to environmental cues (8). For the portion of the tumor sampled here, the path to oncogenic EGFR signaling was relatively linear. Based on shared clonal features in addition to *EGFRvIII*, the authors were able to use a chromothriptic event involving chromosomes 1, 4, 6 to define the point at which a minor subclone split from the major tumor population.

For tumor 2, integrated analysis revealed a very different evolutionary course. Multiple amplified *EGFR*s were detected, including: 1) wild type *EGFR*; 2) *EGFRvII*, which contains a deletion of exons 14 and 15; 3) *EGFRvII*-extended, a novel deletion that extends from exon 14 to include alternative exon 16; 4) a C-terminal *EGFR* truncation and 5) an *EGFR* harboring deletion of exons 25–26. Integrated analysis of these single cell *EGFR* variants with homozygous and hemizygous deletions detected in bulk tumor, revealed a far more remarkable, non-linear and surprising path to oncogenic *EGFR*. Unlike tumor 1, all of the *EGFR* variants were mutually exclusive of wild type *EGFR* amplification and of each other. This convincing demonstration of convergent evolution of independent *EGFR* gain of function variants in a single tumor provides compelling independent evidence for the importance of altered *EGFR* in GBM.

It is possible that intratumoral heterogeneity may be even greater than initially suspected. In some cases like tumor 1, the path to oncogenic *EGFR* is relatively linear, whereas in examples like tumor 2, convergent evolution of independent *EGFR* variants may drive oncogenic signaling. These findings have important clinical implications. Tumor composition may be an emergent property arising from the interplay of competition and cooperation between tumor cells differing in genetic and functional properties. This emergent balance can potentially be shaped by nutrient limitations, regional influences and the capability of tumor cells to signal to each other (7). Personalizing therapies for patients based on molecular composition may therefore depend on being able to detect these mutational hierarchies and use them to guide more effective therapies, including upfront combinations (Fig. 1B).

Like all good studies, this paper raises a number of important questions for future study. The extent of clonal diversity of *EGFR* mutants in GBM, and their functional and therapeutic properties needs to be further characterized. It is interesting to note that *EGFRvII* enhanced sensitivity to EGFR TKIs. The spectrum of *EGFR* variants, and extracellular domain missense mutations will need to be further characterized to determine if these lesions sensitize tumor cells to ATP competitive TKIs, EGFR-targeted antibodies, and or combinations. Further, EGFR variant co-expression and functional interaction with other RTK alterations needs to be further clarified to determine whether multiple RTK inhibitors will be needed (Fig. 1, tumor 3). This study examined samples that were less than 1cm. How diverse are tumor cells across different regions of a tumor?

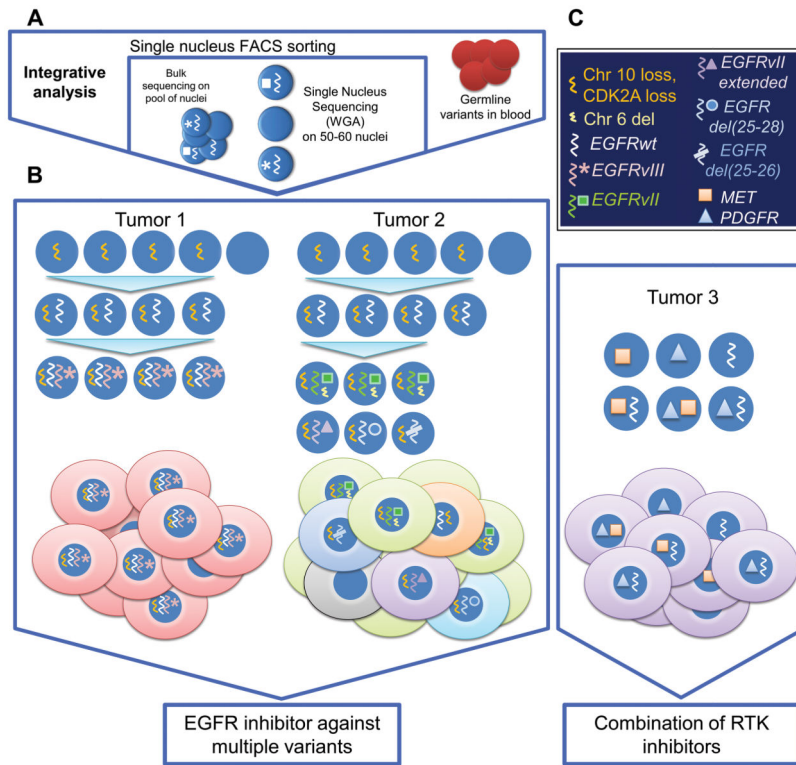
*EGFR* mutations in GBM occur almost exclusively in the extracellular domain of the receptor. In contrast, in tumors from other parts of the body, the *EGFR* mutations occur primarily in the kinase domain of the receptor (4). Is there a similar convergent evolution of independent *EGFR* mutations in these other cancer types? Most importantly, future studies will be needed to assess how targeted treatments alter the molecular composition of tumors, possibly helping to anticipate and develop therapies that suppress drug resistance. The future of personalized cancer therapy will depend on treating tumors as an ecosystem, not as a collection of individual cells. The approach developed by Francis and colleagues (11) provides an important path forward.

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**Figure 1. Integrative analysis of single nucleus whole genome sequencing and bulk tumor tissue enables detection of the subclonal architecture of tumors**  
 A) Schematic view of the analytic approach. B) A schematic view of illustrative GBM cases. Tumor 1 shows linear evolution of a GBM whose cells relatively uniformly contain both amplified wild type *EGFR* and *EGFRvIII*. Tumor 2 shows a far more complex evolutionary path, characterized by emergence of multiple, independent, mutually exclusive *EGFR* variants. Tumor 3 represents an alternative scenario, in which individual GBM cells vary in their amplification of *EGFR*, *PDGFRa* and *MET*. C) Genomic alterations: legend.