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Molecular targeting of Glioblastoma – how do you hit a moving target?

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Abstract

Cancer is a molecularly complex, genomically unstable disease. Selection for drug resistance mutations, activation of feedback loops and upregulation of cross-talk pathways provide escape routes by which cancer cells maintain signal flux through critical downstream effectors to promote therapeutic resistance. Targeting signal transduction pathways in cancer may therefore require aiming at a "moving target". The routes of resistance need to be anticipated to guide the selection of drugs that will lead to durable therapeutic response. In this ***New Strategies*** article, we discuss the challenges imposed by the complexity and "adaptive" capacity of cancer and suggest potential new diagnostic strategies in order to more effectively guide targeted cancer therapy. We focus on glioblastoma (GBM), the most common malignant primary brain tumor of adults. GBM is a model for a "pathway-driven", molecularly heterogeneous cancer for which new genomic insights obtained through **The Cancer Genome Atlas** are ripe for integration with functional biology and incorporation into new molecular diagnostic assays.

Background

THE CHALLENGE OF TARGETED THERAPY

In a 2006 perspective in ***Nature***, Reuben Shaw and Lew Cantley suggest that: "As more drugs that target specific components of signal transduction pathways become available and as we increase our knowledge of the complexity of these signaling networks, the burden of selecting the right drug combinations for each individual cancer patient will ultimately shift to the pathologist who must identify the underlying defect in each tumor"(1). These words crisply embody the vision of personalized cancer medicine; they also impose a formidable challenge. Finding the "underlying defect" becomes a significant challenge when genomic surveys identify a "landscape" of hundreds of mutated genes within each individual tumor.

Further, targeting the signal transduction pathways that a tumor needs in order to proliferate and survive is like trying to strike a moving target. Interconnectivity between oncogenic signaling pathways enables cancer cells to evade targeted therapy by maintaining signal flux to downstream effectors (2–4), through both genetic (5)and non-genetic (6)mechanisms. Selection of cancer cells bearing kinase inhibitor resistant mutations can promote resistance to signal transduction inhibitors, as has been demonstrated for CML patients treated with

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imatinib and non-small cell lung cancer patients treated with erlotinib or gefitinib (7–10). On top of this already robust repertoire of escape tools, radiation and chemotherapy, the front line treatments received by virtually all GBM patients, greatly increases background mutation rates (11), providing ample opportunity for development and selection of therapeutically resistant clones.

The challenges of developing more effective targeted therapies and combination therapy strategies are significant. However, it is also a time of unprecedented opportunity. A confluence of circumstances now makes GBM an ideal model in which to consider the challenges of personalized therapy. GBM is one of the initial cancers sequenced by the TCGA providing a window into its mutational landscape (11,12). Independently, powerful mouse genetic models of GBM have been developed demonstrating the importance of the very same mutations identified by the TCGA in the development and progression of GBM (13–22). In addition, GBM is a paradigmatic example of intratumoral cellular and molecular heterogeneity, for which recent data suggest that tumor cell subpopulations, either as cancer stem cells or through clonal selection, may be important in the development of resistance to therapy (23–25). Finally, the glioblastoma research community is well-organized and highly collaborative, which has facilitated the performance of molecularly guided clinical trials in which the effect of targeted agents on their intended signaling pathways has been quantified and correlated with biological and clinical outcomes. The thesis of this ***New Strategies*** article is that the mutational landscape of cancer will need to be integrated with functional studies, particularly those conducted in patients, in order to translate the molecular catalog into better treatment in the clinic.

We propose three steps to address the challenge of developing more effective personalized therapy for GBM patients. Step 1 builds upon the work of the TCGA to expand and refine the “molecular catalog” of GBM. Step 2 requires development of an interactive dynamic network map of GBM that is heavily informed through studying patients in well-designed clinical trials in which molecular endpoints are carefully analyzed. Step 3 necessitates developing tools that facilitate determination of the effect of drugs on signaling networks in defined tumor cell subsets, with resolution to the single cell level. Developing a better understanding of the functional biology of these interactive pathways, in part through the steps outlined above and through their integration with studies in model systems, will be essential for developing more effective personalized treatments for GBM patients.

STEP 1 - BUILD AND REFINE THE MOLECULAR CATOLOG

The ability to catalog the landscape of somatic DNA mutations in tumor tissue from individual patients may well represent the most important advance towards personalizing therapy. Whole genome sequencing technologies make the possibility of individual cancer genomes both technically and financially feasiblein the near future (26). Genomic surveys of the mutational landscape of GBM have provided an important resource to the community, and have demonstrated that mutations occur most commonly in genes whose protein products regulate “core” signaling pathways that control cell growth(11,27). These studies identify many of the mutations and copy number alterations that are already known to be important in GBM, as well as other types of cancer including EGFR amplification and mutation, PTEN loss, p53 mutation, CDKN2A and CDKN2B loss and mutation (28–30). These surveys also identified previously unrecognized alterations such as NF1 loss and IDH1 mutation (11). Most importantly, the comprehensive integrated approach of these studies demonstrated that the mutations and copy number alterations cluster along “core pathways” including: 1) receptor tyrosine kinase/RAS/PI3K signaling, 2) p53 signaling and 3) pRB signaling. Cooperative co-activation of these core pathways gives rise to GBM in a wide range of mouse genetic models (13–22), providing scientifically gratifying, functional support for the importance of cooperation between these core pathways in the formation,

maintenance and progression of GBM. Integration of diverse types of molecular data, linking transcriptional signatures, methylation patterns and signal transduction pathway profiles with genomic subtypes based on core pathway alterations has therefore opened up a window into a systems biology level understanding of GBM, potentially yielding an array of therapeutic targets (12,31). Additional "core pathways" and their modifiers may soon be uncovered. However, the extent of genomic complexity appears to be daunting. A recent study of the genome at 30x coverage of U87, the most commonly studied GBM in vitro cell line model, revealed a striking degree of complexity; 512 genes were homozygously mutated, including single nucleotide variatons, small insertions and deletions, microdeletions and interchromosomal translocations (54). The challenge of translating this myriad of DNA alterations into drug targets will require not only an intense investment in the bioinformatic infrastructure, but also a commitment to sequence clinical samples, including before and after treatment in patients treated with targeted agents in clinical trials. It will also require a serious commitment to functional experiments in *in vitro* and *in vivo models* to elucidate the targets and to understand the molecular context that determines therapeutic response.

STEP 2 - DEVELOP AN INTERACTIVE DYNAMIC NETWORK MAP IN PART BY STUDYING PATIENTS

Even at this relatively early stage of cancer genomics though, it appears that knowing the mutational landscape, i.e. the catalog of mutations, will not be sufficient for guiding more effective personalized cancer therapy. Despite the compelling biological plausibility, small molecule inhibitors targeting key proteins within these core pathways i.e. the EGFR inhibitors erlotinib and gefitinib and the mTOR complex inhibitor rapamycin and its analogues, have failed to demonstrate clinical benefit for GBM patients, resulting in very few clinical responses of very short duration (32–35). One potential explanation for this disconnect, is that other mutations and core pathways that have yet to be uncovered will prove to be more suitable molecular targets. This is possible. However, the frequency of alterations of the currently identified core pathways, their importance across many cancer types, and their clear role in promoting tumor formation and progression in mouse genetic models strongly suggest that the currently identified candidates are among the most compelling therapeutic targets.

Another possibility arises from the recognition that acore pathway consists of a complex interacting networks of proteins; not single linear pathways. Therefore, therapeutic resistance to small molecule inhibitors may be achieved through cross-talk within and/or between core pathways to maintain signal flux to key downstream effectors, even if the "right" molecules are targeted. Our own work identifying mechanisms of resistance to EGFR inhibitors (35)and that of others (6,36,37) clearly demonstrate this point –maintained signal flux through PI3K appears to be a common denominator for alternative mechanisms of resistance to EGFR-targeted therapies. Therefore, understanding the static "architecture" of the mutationally activated linear pathways is not likely to be sufficient for guiding treatment. Instead, a "map" of the dynamic interactive architecture of the target signaling pathways will be needed in order to develop more effective combination therapies.

Developing a dynamic interactive network map presents a formidable challenge. It will require development of quantitative tools to measure many nodes in a complex signaling network in patient tissue samples, and more importantly, it mandates application of these tools to patient samples treated with targeted agents in the clinic. This requires a significant intellectual and financial investment in developing the infrastructure to measure the engagement of new agents with their intended molecular targets (38) and to assess their impact broadly on signal transduction in patients. In fact, this challenge becomes as essential a component of developing personalized treatment as does illuminating the molecular

catalog of each patient. More sensitive molecular diagnostic tools that can quantify the effect of new agents on different cellular subpopulations within a tumor are needed. Further, a new level of collaboration between clinicians, biologists, translational scientists and bioinformatics/statistics experts in designing clinical trials must be realized in order to make this possible. It will also require an unprecedented level of collaboration between academics and partners in the pharmaceutical industry to help develop clinical trials with molecular endpoints. In the next section, we provide one illustrative example in which we uncovered the "dynamic" architecture of key signaling pathways by studying patients enrolled in a clinical trial.

An illuminating example—Here, we will provide one illuminating example in which only a small subnetwork was studied in a small subset of patients. However, the lessons learned suggest that in-depth molecular analysis of patient samples, and the effect of drugs on their intended pathway targets in carefully designed clinical trials, even of incomplete and limited networks of signaling molecules, may provide critical insights that lead to better treatments. The mammalian target of rapamycin, mTOR, emerged for many reasons as a compelling molecular target in cancer. PI3K signaling is hyperactivated in nearly 90% of GBMs, and mTOR kinase is one of its critical effectors. mTOR exists in two signaling complexes. As part of complex 1 (mTORC1), mTOR is an important downstream effector of PI3K/Akt activity linking growth factor signaling with protein translation and cellular proliferation. As part of complex 2 (mTORC2), mTOR is a critical activator of Akt. Rapamycin and its derivatives are highly effective at blocking mTORC1 signaling. Rapamycin and its analogues have been shown to be remarkably effective in preclinical mouse GBM models (39–41), and they had already been given to patients as an immunosuppressive therapy, generating hope for more effective targeted therapy for GBM patients. However, Phase II studies of single agent rapamycin analog in recurrent glioblastoma multiforme demonstrated no clinical efficacy (33,42). How can the disconnect between the compelling nature of the target and the dismal record in the clinic be reconciled?

We reasoned that for targeted agents that inhibit the activity of specific signaling pathways such as the mTORC1/S6K1/S6 signaling axis, assays to assess adequacy of pathway inhibition in patients need to be incorporated into the design, interpretation and implementation of clinical trials. In a close multidisciplinary collaboration involving clinicians, biologists, translational scientists and bioinformatics/statisticexperts, we conducted a small pilot phase I/II neoadjuvant clinical trial of rapamycin in patients with relapsed, PTEN-negative glioblastomaat three dose cohorts. Salvage surgical resection is often part of the clinical management of glioblastoma patients who relapse after standard up-front therapy (which typically consists of surgical resection followed by adjuvant radiation and chemotherapy). This presented an opportunity to design a molecularly guided clinical trial that included molecular selection criteria and that enabled molecular analysis of the effect of rapamycin on mTOR signaling *in vivo*. Rapamycin was orally administered to patients prior to a scheduled tumor resection with the primary goals of defining a dose required for mTOR target inhibition and assessing potential anti-proliferative effects on tumor cells. Uponinitial clinical presentation and biopsy evaluation, PTEN status within tumor tissue was determined. Upon relapse after standard upfront therapy, patients whose tumors were determined to be PTEN deficient at initial biopsy received a 10 day course of rapamycin, at three dose cohorts (2,5,10 mg/day B.I.D.) followed by surgical excision. Intratumor drug levels, mTORC1 signaling (as measured by S6 phosphorylation) and cellular proliferation were measured and compared between surgery 1 and surgery 2 in rapamycin treated patients, and in a set of similarly matched "control" patients with relapsed glioblastomas who did not receive rapamycin treatment.

Intratumoral rapamycin concentrations sufficient to inhibit mTOR *in vitro* were achieved in all patients, even those at the lowest dose. However, the magnitude of mTORC1 inhibition in tumor cells varied significantly from patient to patient (from 10%–80% inhibition of S6 phosphorylation). Reduction in tumor cell proliferation (as measured by Ki67 staining) in vivo was significantly related to the degree of inhibition of mTORC1 signaling. Inhibition of greater than 50% resulted in significantly inhibited tumor cell proliferation; in contrast, lower levels of mTOR inhibition did not translate into cytostatic response in patients. Further, tumor cells removed from rapamycin "resistant" patients that we cultured ex vivo were found to be highly sensitive to the drug. Thus, resistance to rapamycin was not cell autonomous, but rather that lack of cytostatic response in patients represented a failure of the drug to fully access its target *in vivo*. These results suggest a different interpretation of the clinical failure of rapamycin in glioblastoma patients: ***the lack of efficacy appeared to be a consequence of incomplete inhibition rather than injudicious choice of molecular target***.

mTORC1 is both a ***positive*** regulator of PI3K/Akt signaling from growth factor receptors and a ***negative*** regulator of PI3K pathway activation when signal flux through PI3K is high. This ensures homeostatic regulation of PI3K activity in healthy cells (43). Therefore, de-repression of mTORC1-mediated feedback by treatment with rapamycin could paradoxically result in more rapid clinical progression by promoting PI3K activity in glioblastoma patients treated with rapamycin.

To test this possibility, rapamycin treatment was reinstituted in these patients following surgery and patients were monitored for progression. Strikingly, rapamycin treatment led to Akt activation in seven of fourteen patients, presumably due to loss of negative feedback, which was associated with significantly shorter time-to-progression during post-surgical maintenance rapamycin therapy[43]. These results highlighted the importance of pathway cross-talk in determining response to targeted therapy and suggested a rational next step towards combination therapy (i.e. dual mTOR/PI3K inhibition). As importantly, this study demonstrated that measuring the effect of the drug on its target signal transduction pathway, and on other proteins in the signaling network, is feasible and can provide insight into mechanisms of therapeutic resistance.

STEP 3 – DISSECT INTRATUMORAL CELLULAR AND MOLECULAR HETEROGENEITY

Human cancer is not a homogenous population of cells, but rather a complex interactive microenvironment composed of different cell types (i.e. cancer cells, inflammatory cells, vascular cells, support cells). Interaction may be critical for tumor development, maintenance and resistance to treatment (44). The cancer cells within a tumor also demonstrate considerable morphological, phenotypic and physiological heterogeneity, varying greatly in their biological aggressiveness (45). Cancer stem cell subpopulations (46,47), phenotypic plasticity (i.e. the result of interactions between the cells genotype and the local the molecular signals it receives from the microenvironment, including through epigenetic changes) (45,48) provide complementary non-genetic routes towards increasing intratumoral cellular and molecular heterogeneity (45). Other non-genetic factors appear to profoundly influence the response of cancer cell subpopulations to treatment potentially promoting therapeutic resistance (49). Therefore, identifying the molecular signals in multiple tumor cell subpopulations may be needed in order to more effectively guide targeted treatment (50). In addition, clonal genetic diversity (51), which can arise from the interaction of rare mutation with local selection pressures, provides a powerful heritable component to intratumoral heterogeneity that can promote resistance to targeted therapies. This concept has been powerfully demonstrated by the emergence of resistance promoting BCR-ABL kinase domain mutations in CML patients treated with imatinib and by resistance promoting EGFR T790M kinase domain mutations in lung cancer patients treated with erlotinib and gefitinb (7–10).

Of great potential clinical relevance, work from the Settleman group has demonstrated the transient acquisition of a drug-tolerant phenotype in a low frequency of individual cancer cells within a tumor, which can give rise to tumor cells that are strikingly resistant to diversity of anti-cancer agents (55). The molecular mechanisms underlying this transient drug tolerant phenotype are only beginning to be understood, however, altered chromatin state mediated by the histone demethylase Jarid1A has been implicated (55). Whether this mechanism is generalizible to many types of cancer, and whether it is clinically relevant remains to be determined. However, this important study raises the critical possibility that subpopulations of tumor cells may acquire a resistant phenotype, and that molecular markers could potentially identify those resistant subpopulations. Further studies will be needed to develop more effective ways to target them.

On the Horizon

How do we as a community move forward to translate knowledge of the cancer genome into more effective treatment for patients? In this article, we have proposed three steps.:

Step 1

This step builds on the TCGA resource to extend and more fully integrate the molecular catalog of cancer mutations. Integration of diverse data types on this framework will rapidly refine the catalog to provide a more integrated view. It will also uncover new targets and potentially identify molecularly-defined subsets of patients that may respond differentially to therapy. As the cost of fully sequencing individual genomes falls, it is even feasible to begin to consider the role of genome sequencing as a basis for tailoring therapy for individual patients in the future. The challenges will lie in extracting biologically meaningful pathways and targets, testing their function in appropriate models, developing agents that target them and understanding the cellular context that determines response or resistance. We believe that many of the key pieces for this are already in place. The work will be difficult, time consuming and laborious, but will likely lead to direct impact in the clinic.

Step 2

This step requires a commitment towards development of better molecular diagnostics to measure engagement of drugs with their targets (38) and to quantitatively assess the impact of these agents on dynamic interactive signal transduction networks. A major thesis of this ***New Strategies*** article is that considerably more attention needs to be paid to this step in order to transform this unprecedented knowledge base into improved outcome for patients. This step represents a commitment to uncovering the dynamic interactive network map by studying patients in clinical trials with molecular endpoints. It will require innovative clinical trial design in which patients are molecularly stratified for treatment and for which tissue is obtained both before treatment, during acute exposure to the drug to assess the engagement with the target pathway, and ultimately at the time resistance develops. The logistics of designing such a re trial are challenging. We will need to justify the additional tissue sampling and we willl have to demonstrate a benefit to patients in monitoring the effect of the drug on its intended target. Because such tissue sampling will need to be as low risk as possible, smaller, less invasive biopsy approaches will be needed, necessitating improved ability to extract maximal molecular information from very small samples. Finally, new molecular diagnostic tools for quantitative, highly multi-plexed, multiparameter measurement of signaling networks will need to be incorporated into the design, implementation and interpretation of clinical trials. Although quite difficult, this step presents an ideal focal point on which to build new types of highly integrated collaboration

between clinicians, biologists, translational scientists, physical scientists and bioinformatics experts, and an ideal point of collaboration between academics and industry.

Step 3

Thjs step necessitates developing technologies that can measure molecular signals in tumor cell subsets with resolution to the single cell level and gauge the effect of new therapies on tumor cell subsets. Integration of tools arising from the physical sciences with genomic platforms and for the analysis of tumor tissue from patients treated with targeted agents in clinical trials will augment our currently existing technological platforms (i.e. phospho-flow cytometery (50) and or application of FISH and immunohistochemistry to clinical samples (45)) to a) greatly increase our understanding cancer as an adaptive microenvironment; b) help identify mechanisms by which subsets of cells drive therapeutic resistance and c) provide a tool that could help develop the “dynamic interactive map” to more effectively guide cancer treatment. A natural integration of steps 1–3 will help provide a better functional understanding of the biology of these interactive pathways. It will be critical to integrate these insights with studies in appropriate models to uncover how signaling networks dynamically interact. Integrating siRNA and small chemical screening library approaches in the context of genetically defined models (particularly ones that reference the molecular subsets being developed through the TCGA) to identify synthetic lethal interactions and illuminating the molecular linkages by which oncogenic signaling regulates altered cellular metabolism (52,53)will greatly enrich our understanding of the biology of the disease, and are likely to provide critical therapeutic leads that can be translated into clinical benefit. In summary, this is a time of unprecedented opportunity and challenge. Disentangling the complexity of cancer; learning the “rules” of complex interactive signaling networks and learning how best to treat the disease by studying patients are all in our grasp. Integration is key; there is much work to be done, but the map is already becoming clearer.

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