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Engineering multi-dimensional evolutionary forces to combat cancer

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Abstract

With advances in technology and bioinformatics we are now positioned to view and manage cancer through an evolutionary lens. This perspective is critical as our appreciation for the role of tumor heterogeneity, tumor immune compartment, and tumor micro-environment on cancer pathogenesis and evolution grows. Here, we explore recent knowledge on the evolutionary basis of cancer pathogenesis and progression, viewing tumors as multi-lineage, multi-component organisms whose growth is regulated by sub-component fitness relationships. We propose reconsidering some current tenets of the cancer management paradigm in order to take better advantage of crucial fitness relationships to improve cancer patient outcomes.

Introduction

Genetic, epigenetic, and non-Darwinian adaptive evolutionary processes act in concert to shape biological landscapes and phenotypes across both physiologic and pathological disease states, including cancers. At their most basic level, cancer cells are normal cells that have acquired somatic genetic alterations and epigenetic changes and no longer follow their physiologic development plan. Acquisition of certain alterations initiates an evolutionary process that results in cellular adaptation and malignancy, the evolutionary trajectories of which are shaped by multiple forces that fuel cancer initiation and progression. These aberrant cells can diverge from the destined program by a multitude of mechanisms, including mutations in the DNA during replication, errors in the DNA replication, repair machinery, and exposure to mutagens. Cancer cells manifest and exploit canonical hallmarks of malignancy to persist (1,2). An additional dimension of complexity is overlaid on tumor cell biology when the contributions from an evolving tumor immune compartment and

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tumor microenvironment are considered. Micro-environmental changes in both immune and stromal compartments, epigenetic influences, and changes in the signaling patterns within and between cells impact tumor cell survival (Figure 1). The state(s) and role(s) of these compartments evolve during treatment and are frequently intertwined (Figure 2).

The evolution of cancer cells is critical for their survival in the complex and competitive niche environments in which they establish themselves either as primary tumors or metastases. It has become clear that there is significant heterogeneity within and between tumor deposits (3). This realization has resulted in a new understanding of the importance of the clonal and subclonal changes and the tumor micro-environment that can drive tumorigenesis and modulate responses to therapy. Additionally, non-genetic cellular phenotypes are influenced by signaling pathway activation and dynamic adaptation (via post-translational regulation) and impact cancer evolution and drug resistance (4).

Herein, we review the basic tenants of cancer evolution as well as our understanding of how the complex relationship between the tumor microenvironment and tumor and native cells evolves. Further, we explore different models and theories for engineering this multi-lineage evolutionary process in a controlled and pro-active manner to create opportunities for improved cancer management and treatment.

Characterizing the Genetic Basis and Heterogeneity in Cancer

Early efforts to characterize somatic mutations in human cancer genomes were limited by the available technology and analytics. Protein kinases were a natural focus given the efficacy of anticancer treatments aimed at protein kinases. Studies evaluating somatic mutation burden in the kinomes of colon cancer (5), breast cancer (6), gliomas (7), and testicular germ cell cancer (8), and multi- or pan-cancer analyses, demonstrated that the majority of somatic mutations in cancer are “passenger” mutations that are not active in driving the cancer (9).

As technology advanced, larger studies yielded additional candidate genes involved in cancer development, and more therapeutic targets, and pushed the evolution of bioinformatic approaches to counter the challenges of false discovery (10). These studies illustrate that oncogenic alterations are often differentially represented in distinct cancer types (11). A theme that emerged from a multitude of cancer genome sequencing efforts is that while somatic mutation burden and specific oncogenic driver genes are often inconsistently present across tumor types, common pathways are dysregulated. Thus, in cancer certain pathway traits are selected for from the genetically-rich substrate of the human genome.

In breast and colon cancer, Wood and colleagues comprehensively characterized the genomic landscape of 22 breast and colon cancers and illustrated the magnitude of the genomic landscape, highlighting the common features between cancers and that there are a finite number of pathways that become dysregulated during tumorigenesis (12). This work provided additional examples illustrating that cancer is a disease of pathway dysregulation with multiple genetic interactions converging on the dysregulation of a set of core pathways (13). In non-small cell lung cancer (NSCLC), Imiulinski et al. sequenced the whole genomes

and/or exomes of 183 lung adenocarcinomas and matched normal adjacent tissue pairs (14). They reported a mean somatic mutation rate of 12 Mutations/Megabase (Mut/Mb). They re-verified 19 established oncogenic driver genes, with overlapping pathways, and identified an additional 6 potential oncogenes of new interest.

Larger, systematic mutational characterization followed in multiple cancer subtypes through the efforts of the Cancer Genome Atlas Research Network and other groups and have resulted in characterization of lung adenocarcinoma, small cell lung cancer, head and neck cancer, multiple myeloma, CLL, NHL, ovarian, colon cancer, and squamous cell lung cancer and glioblastoma multiforme (10,15–23). Through these efforts, putative cancer genes and their requisite pathways were identified within and between multiple malignancies and the challenge of tumor genomic heterogeneity was magnified. Multiple co-existing genetic alterations contributing to tumor evolution were often present in individual tumors and patients (10,15–26).

In landmark studies, two research teams characterized the mutational spectrum and heterogeneity within and between cancers. Published contemporaneously, these datasets demonstrate the complexity and depth of cancer somatic mutations. Lawrence MS, Stojanow P et al. evaluated the heterogeneity in mutation rates in a dataset of 3,083 tumor/normal pairs across 27 tumor types (27). This work identified two primary contributors: first, the importance of gene expression level; the mutation rate is correlated to gene expression level and replication time due to transcription-coupled DNA repair processes and second, DNA regions that are replicated later in the cell cycle have higher mutation rates. In parallel work, Alexandrov LB et al. clarified the challenging and sometimes divergent results of a multitude of prior studies by exploring mutational frequency across different cancer subtypes (28). They identified and classified nearly 5 million somatic mutations by comparing tumor versus normal tissue in 7,042 cancers. They determined that there was a high variability in the somatic mutation burden, ranging from 0.001 Mut/Mb to >400 Mut/Mb and demonstrated that cancers related to chronic mutagenic exposures exhibit the highest prevalence of somatic mutations. Further, they identified 21 distinct mutational signatures based on DNA base substitution classification and their application to the 30 cancer subtypes. Interestingly, they also identified localized regions with hypermutation, or *kataegis*. It is thought that a subfamily of the APOBEC (apolipo-protein B mRNA editing enzyme, catalytic polypeptide like) cytidine deaminases may be a causative factor in genomic regions with *kataegis* due to the similarity in the mutational signature (29). In breast cancer, *kataegis* signatures correlate with favorable prognosis and in lung cancer the APOBEC mutational signature, appears to underlie tumor subclonal diversification and be a predictive marker of response to immunotherapy (26,29,30).

Longitudinal and Evolutionary Views

To further investigate the interplay of pathway alterations and heterogeneity of tumors, several groups have developed large scale data monitoring projects to characterize and track intratumoral heterogeneity during treatment. Studies addressing this critical evolutionary question include breast (31,32), pancreatic (33,34), kidney (3), colorectal (35,36), prostate (37), lung cancers (38), urothelial cancers (39,40) and CLL (41). In metastatic renal cell

cancer, Gerlinger et al. evaluated intra-tumoral heterogeneity in their pioneering study by analyzing tumor tissue from primary and metastatic disease sites within the same patient. This study provided a new appreciation for the complexity facing targeted cancer therapy, given that 63–69% of mutations identified were not found in all individually assayed samples from the same patient. The heterogeneity was present in all levels of analysis; genome, transcriptome and proteome (3).

In colorectal cancer, multiple studies have characterized heterogeneity within and between primary and metastatic disease sites as well as the evolution of advanced-stage cancers (42–47). These studies were critically important in demonstrating that there is not necessarily concordance between the primary tumor and metastatic site, even when sampled synchronously, and that in colorectal cancer clonal evolution can be tracked, as in other disease types (44,45,48,49). Also in colorectal cancer, cell-free DNA (cfDNA) was utilized to monitor the molecular evolution of acquired resistance and to overcome the challenge of tumor metastasis heterogeneity given that it is a sample of tumor DNA that is representative of the larger disease burden. Diaz et al. utilized cfDNA to demonstrate that *KRAS* wildtype patients treated with panitumumab can selectively evolve a resistant subclonal population and further that these resistance mutations are present before treatment. These findings highlight the importance of understanding baseline tumor heterogeneity and its dynamic evolution (36). Landmark work in colorectal cancer showed the utility of monitoring the dynamic changes and responses of intermittent drug schedules, as well as the value of assessing cfDNA to monitor treatment response and the emergence and regression of resistant subclones (50,51). Russo et al. demonstrated that utilization of molecular response data can inform treatment selection as new resistance-driving clones arise (51).

In high-grade serous ovarian cancer, Bashashat et al. profiled 31 spatially and temporally parsed ovarian cancer specimens obtained from 6 patients. Only a portion (51.5%, range 10.2–91.4%) of the mutations were present in each sample from a given patient (52), reinforcing the presence of extensive inter-tumor genomic heterogeneity within individual patients.

In lung cancer, Jamal-Hanjani et al reported on several aspects of the first 100 of an anticipated 842 early stage patients in the Tracking Cancer Evolution through Therapy (TRACERx) program (53,54). They found that a median of 30% of somatic mutations are subclonal and 48% of copy number alterations are subclonal, indicating that mutational processes are ongoing during tumor development. They further evaluated the timeline of genetic alterations and demonstrated the impact of chromosomal instability, which was the primary driver of parallel evolution (55). In a followup analysis, they explored allele specific human leukocyte antigen (HLA) loss and immune escape in the same cohort of patients and showed its presence in 40% of early-stage NSCLCs. The work also demonstrated that this is an evolutionarily-selected process associated with increased non-synonymous mutations and higher neo-antigen burden. Analysis of patients with stage IV disease demonstrated that this process occurs preferentially at metastatic sites (56). These findings link tumor genomic alterations and heterogeneity to immune escape and are parallel to findings in melanoma in which loss of heterozygosity in major histocompatibility complex class I antigen presentation components may contribute to resistance to certain immunotherapies (57).

Highlighting the genetic complexity present within individual tumors and patients, Blakely et. al evaluated 1,122 EGFR positive cfDNA samples from patients at different time-points during systemic therapy. They demonstrated in a subset of patients with repeated assays during treatment that the tumor genomic complexity increased during treatment with an increasing number of somatic mutations (38). This observation suggests that in this disease treatment with a targeted therapy does not result in a purified genetic landscape, but instead may increase genetic diversity that contributes to ongoing tumor evolution and adaptation.

In renal cell carcinoma three topical analyses were published simultaneously (58–60). Turajlic et al evaluated 1,206 tumor regions from 101 patients and 106 tumors (all stages). Using a combination of driver panel sequencing, whole exome sequencing and whole genome sequencing, the group identified 740 somatic mutations. Correlation of non-synonymous mutations with known driver mutations identified a median of 3 driver mutations per tumor. They also found that von Hippel-Lindau related alterations were the only consistent clonal event, present in 95 of 106 tumors. Subclonal alterations (copy number alterations and mutations) were associated with known prognostic markers and patient outcome (59). The group analyzed 575 primary and 335 metastatic biopsies in 100 patients with metastatic renal cancer to characterize heterogeneity in metastatic versus primary (localized) disease. Their compelling findings demonstrated that the overall number of non-synonymous mutations and copy number alterations were more prevalent in the primary tumor than the metastasis. Further, they demonstrated that most genomic diversity was present in the primary tumor while only 5.4% of the genetic alterations arose *de novo* in the metastatic tumor site, reflecting an evolutionary bottleneck. Interestingly, as a parallel to the TRACERx lung findings, TRACERx renal also observed an increased imbalance at the HLA locus in selected clones (54,58).

The TRACERx prostate and melanoma groups have identified equally compelling data regarding intra-tumoral heterogeneity in prostate cancer and an evaluation of the tumor microenvironment in melanoma (61,62). A balanced focus on both the tumor and the microenvironment is critical given our increasing appreciation of the importance in the tumor microenvironment in modulating response to treatment and selective pressure from the microenvironment impacting tumor evolution.

A unifying theme across these studies is the presence of substantial diversity in populations that increases during the selective pressure of treatment and that this heterogeneity can be monitored over time. With targeted therapies, molecular heterogeneity dictates treatment response; however, tumor cells do not exist in isolation and the next frontier in understanding tumor biology and identifying treatment strategies requires a more comprehensive understanding of not only how tumor cells evolve with different treatments but also how the entire tumor ecosystem interacts and shifts in composition and cell state(s) during therapy.

Insights from Single Cell Organisms and Hematologic Malignancies

Many parallels to these findings have been identified in single cell organisms in which evolutionary studies are more feasible. For example, in yeast, steady state cultures of

different yeast strains grown in chemostats can be monitored for hundreds of generations to characterize and manipulate evolution and apply selective pressure. Hope et. al demonstrated this by characterizing the frequency of different genetic pathways leading to an aggregation phenotype called flocculation (63). In these experiments, they identified the specific frequencies of the most common genetic changes leading to aggregation and determined that there was a favored evolutionary path to this trait, as opposed to several equally evolutionarily favored routes. Further, they characterized the most favorable adaptive route in different genetic backgrounds and the consequences of blocking it, which ultimately delayed, but did not prevent the development of the flocculation phenotype and revealed alternative pathways for phenotype development (63).

These results parallel the development of the EGFR T790M resistance mutation in EGFR mutated NSCLC when treated with a first or second generation TKI (64). There are several known mechanisms of resistance to first and second generation EGFR TKIs, but the most common mechanism is via the secondary mutation, T790M. When EGFR signaling is effectively blocked, drug resistance is attenuated, but the cancers evolve and find other routes to become resistant. This occurs regardless of oncogenic alteration or the targeted therapy. Separate from secondary mutations, bypass pathways can also become activated, such as various RTKs and MAPK and PI3K pathway components, that permit tumor progression despite effective blockade of the primary oncogenic target. On-target and off-target resistance mechanisms to targeted therapies in NSCLC have been reviewed in detail elsewhere (65). In colon cancer bypass pathways via biochemical requiring have also been identified. In BRAF V600E mutant melanoma, resistance to BRAF/MEK inhibitor treatment can occur through EGFR expression that is acquired through treatment. A mechanism of biochemical rewiring and resistance is via activation of the EGFR pathway by suppression of SOX10 and activation of TGF- β (66–68).

In bacteria, the evolutionary pressure of antimicrobials results in resistance via numerous different mechanisms, both intrinsic and extrinsic factors have been well characterized. Furthermore, the process of evolution in bacteria has been manipulated to evolve collateral sensitivity in which bacterial strains are designed to become more sensitive to subsequent treatments, based on prior treatments. Imamovic and Sommer demonstrated this in *Escherichia coli* with drug cycling protocols that avoid development of resistance using sensitivity profiles (69). Yen and Papin built on this work by investigating whether prior treatment constrains or potentiates response to subsequent treatment. They demonstrated that they could impact the evolution of *Pseudomonas aeruginosa* by demonstrating drug-order specific evolution and mutations. They not only observed different resistance levels of specific drugs based on prior treatment exposures but also identified genetic mutations and epigenetic processes underlying these responses (70). These findings have strong parallels to cancer biology and the idea of combinatorial and serial therapy designed to evolve tumor cells with collateral sensitivity. Furthermore, they demonstrate that there is ongoing inter-clone competition that evolves based on exposure to treatment and even environmental factors. This has been demonstrated in malignancy as well; in multiple myeloma, Keats et al. illustrated this point in a patient with two competing sub-clones that alternated dominance multiple times based on treatment until one clone underwent a dramatic linear evolution (71).

Another parallel between cancer biology and single cell organisms is with quorum sensing, in which bacteria use small secreted molecules (autoinducers) to facilitate interspecies communication and impact virulence factors, biofilm formation, and alter the microenvironment to interfere with other species by altering native flora and thus impact susceptibility to pathogens (72,73). Autoinducers and their receptors are important targets for disrupting pathogenic processes and an active area of research (74). The corollary in cancer is the secretion of factors by therapeutically stressed tumor cells or the micro-environment that can support tumor cell survival (75–77).

Though more complex than single cell organisms, hematologic malignancies contain fewer somatic mutations than most solid tumors (28). It is interesting to consider whether this translates to more robust responses to targeted therapies. In the solid tumors with the relevant genetic alteration, the complete response rate with targeted therapy (e.g. dabrafenib plus trametinib against mutant BRAF, osimertinib against mutant EGFR, and alectinib against ALK gene rearrangements) is low (<10%) (78–80). In hematologic tumors, the determination of response is measured differently; in CML, complete molecular response (CMR) and major molecular response (MMR) are similar counterparts to complete and partial response in solid tumors. The maximal rate of CMR is 20%, while the maximal MMR is 75% for nilotinib and the maximal CMR is 7% and 87% MMR for dasatinib (81,82). In patients with relapsed/refractory myeloid malignancies with mutant IDH2 treated with the IDH2 inhibitor enasidenib, the complete response rate was 20% while the rate of incomplete response was 18%(83). Further studies of AML patients treated with enasidenib demonstrated on a single cell level that resistance occurred through multiple mechanisms involving epigenetic or genetic mechanisms that promoted the differentiation arrest that was reverted on initial treatment (84).

There are several parallels between solid tumors and hematologic malignancies and a trend toward more complete responses in patients with hematologic malignancies treated with targeted therapies. The mechanisms of resistance are similar in that both off and on target mechanisms of resistance develop in clonal populations (85). Despite the appealing concept that clonal evolution in hematologic malignancies should follow a linear evolutionary trajectory given that the tumor cells are not compartmentalized, as in solid tumors, they actually have been found to frequently follow a branching evolutionary path. This implies that complex clonal interactions are ongoing and likely include tumor, immune, epigenetic and micro-environmental influences (85). The challenge is how to take advantage of this complexity and whether it is possible to stimulate an intra-tumoral fitness competition. For instance, different tumor cell subclones present with a tumor might act as cooperators or antagonists, with distinct therapeutic implications in each case (Figure 2). An interesting question for future study is whether the character or abundance of different subclonal tumor cell populations can be therapeutically controlled to engineer overall constraints on tumor progression or drug resistance.

Immunotherapy and treatment response evolution

Immunotherapy (immune checkpoint blockade) has revolutionized the treatment of many cancers. Unlike targeted therapies in which tumor cells develop resistance by acquiring

mutations and/or activation of bypass signaling pathways, in immunotherapy, the tumor clonal landscape is changed due to complex interactions involving multiple factors that are impacted by the clonal composition and heterogeneity of the tumor (86,87).

The anti-tumor mechanism of action of immunotherapy is indirect compared to chemotherapy and targeted therapy. The complexity of the immune compartment and its interactions with different cell types is reviewed in detail elsewhere (87). Due to its integral role, it is important to characterize how immunotherapy impacts the tumor immune compartment, tumor cells and the tumor microenvironment. Preclinically, Li et al demonstrated in pancreatic adenocarcinoma mouse models that tumor cell intrinsic production of the chemokine CXCL1 modulated sensitivity to immunotherapy (88). Clinically, Riaz et al. characterized the genomic changes in the tumors of 68 patients with melanoma after progression of immunotherapy (89). In doing so, they identified a complex network of relationships between the host immune system, the tumor microenvironment and tumor cells. They described an association between mutational burden and response to therapy in treatment naïve patients that was not observed in patients who were previously treated with ipilimumab (an anti-CTLA4 antibody). They also demonstrated that after 4 weeks of nivolumab (an anti-PD-1 antibody) therapy there was a significant decrease in detectable mutation and neoantigen burden in patients with complete or partial tumor responses, but only a modest decrease in patients with stable or progressive disease. Further, they demonstrated that the tumor genetic clonal landscape became more contracted in patients whose tumors responded to treatment. They also evaluated the tumor microenvironment and demonstrated a correlation between T-cell expansion in proportion to neoantigen depletion in patients who were immunotherapy naïve (90). Interestingly, Jerby-Aron et al. recently demonstrated the ability to identify a gene expression signature predictive of immunotherapy treatment response in melanoma and how the immune response can be modulated by CDK4/6 (91). In NSCLC, Anagnostou et al demonstrated that after treatment with immune checkpoint inhibitors mutation-associated neoantigens are eliminated, thus altering the genomic landscape of tumors (92). In addition to neoantigen exposure to assist with immune targeting of tumor cells, immune-mediated tumor cell death requires activation of T-cells. This activation is also partially facilitated by activation of multiple epigenetic mechanisms; combining epigenetically targeted therapies and immunotherapies has the potential for significant clinical impact (86). Indeed, there are numerous ongoing efforts to determine the feasibility and efficacy of this emerging polytherapy strategy (93).

Epigenetic Landscape

Lineage heterogeneity as well as plasticity are important features of many cancers. Plasticity is enabled, in part, by the epigenetic landscape of tumor cells and its malleability during tumor progression or under drug therapy. Epigenetic influences refer to changes in gene expression and cellular function and phenotype without permanent changes in the genome. Many epigenetic mechanisms can impact cellular functions and can promote or inhibit tumor cell growth when dysregulated. Commonly recognized mechanisms include DNA methylation, and histone or chromatin modifications. While numerous studies have addressed somatic genetic heterogeneity of tumor cells, characterization of the heterogeneity

of the epigenetic landscape is less well explored. The heterogeneity observed in tumors in response to treatment is a combined reflection of the heterogeneity of the tumor cell DNA, the micro-environment and the epigenetic forces shaping cell states and tumor evolution. Epigenetic influences can provide selective advantages to cancer cells given that epigenetic modifications can be applied and regulated on a more rapid time scale than genomic evolution (94).

These epigenetic mechanisms can result in the persistence of cancer cells during treatment and ultimately limit responses to treatment by facilitating resistance. Evidence of “persister” tumor cell populations has been previously discussed and their transient and reversible nature is a natural fit for the properties of epigenetic regulation. Sharma et al elucidated these drug tolerant persister cells elegantly in a NSCLC cell line and identified potential mechanisms by which to target them via IGF-1 and HDAC inhibitors (95). In BRAF V600E cancers, the ERK pathway is rapidly modulated in response to treatment (96). Unlike in melanoma, the clinical response to BRAF/MEK inhibitor treatment in BRAF V600E mutant colon cancers is relatively limited (97). One mechanism of this resistance is thought to be under epigenetic control via activation of the PI3K/AKT pathway (98).

Epithelial to mesenchymal transition (EMT) is another example of an adaptive, epigenetically influenced cellular process in which epithelial cells undergo a transition and lose polarity and cell-to-cell contacts and become resistant to apoptosis (99,100). In normal cells EMT is a critical component of wound healing and embryonic development. In tumors, EMT is thought to contribute to metastasis and treatment resistance (100). EMT is induced by transcription factors responding to extracellular cues such as TGF- β (100–102). Importantly, EMT is not a binomial state and instead is a spectrum that allows cells to adapt quickly to new environmental cues to maximize survival. In malignancy, EMT is often induced by receptor tyrosine kinase inhibition, resulting in adaptive selection for cells that have transition to EMT (100). In NSCLC, specifically this is a well explored mechanism of resistance to EGFR TKI treatment (103–105).

Beyond EMT, the utility of leveraging epigenetics in the management of cancer is apparent in the case of DNA methylation of the O-6-Methylguanine-DNA Methyltransferase (MGMT) gene in glioblastoma. The normal function of this gene is to repair DNA damage (106). When methylated in the promoter, MGMT gene expression is silenced and the cells have impaired DNA damage repair and are more susceptible to DNA damaging chemotherapy agents such as temozolomide. Patients with a methylated MGMT promoter demonstrated a survival advantage to those without (106). Conversely, DNA methylation of promoter regions in other genes can alter gene expression and drive resistance to chemotherapy, as observed in NSCLC and ovarian cancer (107–110).

Additional mechanisms of epigenetic alterations include histone modifications and micro-RNAs (miRNAs). Histone modifications have become a frequent area of focus and include histone acetylation, deacetylation, and methylation. Several histone deacetylase (HDAC) inhibitors are currently in clinical use in multiple different cancer subtypes with additional clinical trials ongoing (111,112). In NSCLC, EMT is impacted by histone acetylation (101). Several studies have demonstrated that HDAC inhibitors can also augment the antitumor

effects of the EGFR TKIs and re-sensitize EGFR TKI resistant cells (113–118). Similar findings have been described in melanoma, in which BRAF inhibitor sensitivity was restored when an HDAC inhibitor was combined with a BRAF inhibitor (119). Furthermore, HDAC inhibitors can increase immune activation by upregulating the expression of MHC class I and II proteins as well as multiple costimulatory molecules (120). In the clinic, the combination of an EGFR TKI and HDAC inhibitor is tolerable; however, efficacy is unclear given the lack of phase II and III clinical trials and HDAC inhibitor (and other epigenetically active drugs)/immunotherapy drug combinations are being tested (93,121–123). Importantly, combinations such as these may need to be deployed in the first line treatment setting instead of after drug resistance fully develops since drug resistance is often multi-factorial in nature.

Micro-RNAs are another type of epigenetic mechanism that is more recently recognized. They are small endogenous non-coding RNA segments that can regulate gene expression, and inhibit or promote cancer by targeting messenger RNAs for activation or de-activation (111,112). Multiple preclinical studies have demonstrated their potential to both monitor therapeutic response and as a therapy target (124). As with other epigenetic influences specific miRNAs can also promote EMT (125,126)

Ultimately incorporating epigenetically-directed treatments could address a major component of tumor plasticity and thereby help to constrain tumor evolution under treatment. These treatments must be studied in different disease settings to understand the best strategy for clinical implementation to blunt this aspect of the plasticity shaping tumor evolution.

Tumor Microenvironment

The tumor microenvironment (TME) consists of the supporting structures and cells that surround and encase cancer cells (127). The TME includes numerous structures such as the vasculature, nervous tissue and extracellular matrix that is comprised of several cell types such as fibroblasts, macrophages, lymphocytes, endothelial cells and the biological materials they generate and secrete. As with epigenetic influences, the TME can inhibit or promote cancer growth. Its composition differs depending on the tissue type and a multitude of other factors. Prior to the identification of targetable oncogenes, a large focus area of cancer biology was the impact of the TME and how it can be manipulated to aid in the treatment of cancer (128). A classic example of this focus is development of angiogenesis inhibitors that alter the tumor microenvironment and modify (inhibit or correct) tumor blood and nutrient supply (129–131). Other examples include efforts at targeting hypoxia in the TME with agents that act upon hypoxia-inducible factor (HIF)-1 α signaling in different types of cancers, and the approval of proteasome inhibitors which appear to act, in part, through re-programming certain pro-tumorigenic features of the TME in multiple myeloma and mantle cell lymphoma (132).

A critical and abundant component of the TME is fibroblast cells. These cells are compelling in their dual role, with both pro and anti-tumorigenic effects. In their anti-tumor state, native fibroblasts regulate the continuous remodeling of the extracellular matrix and

can suppress cancer cell growth by contact inhibition and contact-independent suppression via secretion of soluble factors such as TGF alpha and beta, TNF, and IL6 (133–138). Conversely, cancer associated fibroblasts (CAFs) trans-differentiate from multiple different stromal cell types via several different epigenetic pathways (138). CAFs are often pro-tumorigenic and permit invasion and facilitate stromal dissemination and metastasis of cancer cells as well as produce numerous cytokines, chemokines, matrix metalloproteinases, other soluble factors that promote cancer growth and suppression of the host immune system (138). Many of these activities are known to directly induce resistance to cancer treatments through these and other paracrine mechanisms (139–142). Given their significant role in tumor surveillance and progression and metastasis, efforts are aimed at targeting this aspect of the TME; however, clinical activity to date is less than compelling for the tested drugs (143,144). In the preclinical setting, there is potential for targeting CAF nuclear receptors with small molecules; using this strategy increased sensitivity to cisplatin in tumor xenograft models (145). While other groups demonstrated promising targets such as the *NRG1/HER3* pathway, *NOX4* and *SerpinB2*, the clinical benefit is under investigation (145–147).

Tumor-associated macrophages (TAMs) are involved in tumor cell survival and may exhibit prognostic implications given pre-clinical evidence that macrophage-derived growth factors can activate multiple cancer promoting pathways in cancer cells in a paracrine manner within the TME (148). In multiple cancer subtypes, TAMs are involved in the EMT by secretion of multiple factors that promote this cell state transition(149).

With the identification of targetable oncogenes, research on the impact of manipulating the microenvironment in tumors with targetable oncogenes slowed. With the advent of clinically effective immunotherapy and the increased understanding of paracrine, pro-tumor cell signaling events emanating from different cell types within the TME, the importance and impact of the TME on survival, tumor progression, and therapy resistance is a renewed, important area of focus.

Tumor Energy Metabolism

A major current challenge is to identify strategies for integrating our knowledge of targetable oncogenes, tumor heterogeneity, tumor drug resistance and the influence of the TME in this setting. A further layer of complexity that we must incorporate is that each of these factors is dynamic during tumor growth and treatment. We must consider the biology of a tumor as an evolutionary process involving population dynamics and competing, or cooperative subpopulations of cells (including tumor, stromal, and immune cells). Work by Ibrahim-Hasim et al. demonstrated this principle *in silico*, *in vitro*, and *in vivo* and showed that small environmental perturbations of the TME extracellular pH can alter the balance of different cancer cell subtypes. This resulted in guided selection of either more invasive aerobic glycolysis phenotypes, or less invasive angiogenic tumor cells depending on the perturbation (150). This work demonstrated that different cancer models (prostate and breast) utilize identical cellular adaptive strategies. These findings highlight the need to renew investigative focus on the TME and the manipulation of the metabolic pathways of the tumor and TME. An ongoing question is whether these types of environmental controls exist

in all types of malignancies and how to integrate them into preclinical experimental systems and clinical diagnostic and therapeutic modalities.

A frequent therapeutic focus of tumor biology is aerobic glycolysis. It has long been appreciated that cancer cells are more reliant on aerobic glycolysis than oxidative phosphorylation, commonly known as the “Warburg effect” (151–154). The challenge is how to leverage this information to treat cancer. There is precedent for the feasibility of targeting metabolism across cancer subtypes and many oncogenic pathways stimulates glycolysis and promotes a more tumorigenic and invasive cellular environment. Mutations in *KRAS*, *PIK3CA*, and *AKT* can activate the AKT-mTOR pathway, which among other activities, promotes glycolysis (155). The tricarboxylic acid (TCA) cycle has also been targeted due to a significant association with diverse malignancies. The enzymes succinate dehydrogenase and fumarate hydratase can dysregulate the epigenomic landscape via alteration of metabolic processes in several cancers, including renal cell carcinoma (156), gastrointestinal stromal tumors (157,158), pheochromocytoma, and paragangliomas (159,160). Another example, also from the TCA cycle, is the presence of mutations in isocitrate dehydrogenase (IDH) isoforms 1 and 2 which result in accumulation of high levels of the onco-metabolite 2-hydroxyglutarate (161,162). The first IDH2 inhibitor was approved by the US Food and Drug Administration in 2017 for use in IDH-mutated AML based on positive clinical trial findings (83).

Epigenetic mechanisms that modulate the Warburg effect were identified in multiple cancer subtypes. Interestingly, many of these mechanisms are via miRNA function and demonstrate the range of pro- and anti-tumor activities of these small nucleotides sequences. For example, the tumor suppressor miRNA miR-15b-5p is downregulated in osteosarcoma, which results in increased aerobic glycolysis (163). In lung cancer models, miR-31-5p can enhance glycolysis and cell proliferation(164). Conversely, in hepatocellular carcinoma miRNA-129-5p blocks glycolysis by targeting pyruvate dehydrogenase kinase 4 (*PDK4*), thus slowing carcinogenesis(165).

After the disappointing realization that due to tumor heterogeneity and evolution on treatment, targeting individual signaling pathways such as the PI3K/AKT/mTOR module generally proved suboptimal clinically, many groups are investigating rational combination therapies to improve the depth and duration of response in patients. The integration of metabolic, epigenetic and cellular programs and their heterogeneity and plasticity is a challenge to address, particularly in the context of tumor evolution during therapy (166). The potential to alter current treatment paradigms to a more “organismal-centric” strategy to control these different sources of heterogeneity and adaptability in cancers offers hope for blunting tumor evolution.

Clinical Trial Limitations and Opportunities for Innovation

One of the challenges in identifying rational combination therapies has been the traditional strategy of drug approval. As drugs are developed, the standard practice is to first determine the maximum tolerated dose (MTD), and then to proceed with efficacy assessments utilizing the MTD. The goal is to deliver the maximum amount of drug to kill cancer cells while

avoiding toxic side effects. However, as noted by Gallaher et al., this strategy was developed before the full appreciation of molecular tumor heterogeneity and relies on the hypothesis that cells that are resistant to a given treatment are not present in the treatment naive population (167). We now recognize that tumor heterogeneity is a critical component of the development of resistance, demonstrating that one of the basic assumptions used for clinical trial design may be flawed. Multiple studies demonstrated equivalent outcomes in patients who received modified regimens of chemotherapy and targeted therapy with decreased dose intensity (168–170). This is particularly important to re-evaluate when considering combination therapies or switch therapies in which toxicity from combined agents may limit combination therapy options. A more adaptive approach may improve outcomes in certain cancer types. Several groups are modeling adaptive therapies, and in prostate cancer adaptive therapy approaches have demonstrated promising results (171). Clinical trials in multiple cancer subtypes are ongoing (NCT03630120, NCT03543969, NCT03511196, NCT02415621). It is critical to explore different drug treatment strategies that take advantage of our knowledge of multiple sub-populations of cancer cells to pro-actively engineer tumor evolution to a more indolent or drug sensitive state, or to drive the cancer into an evolutionary pause or “dead-end”.

In breast cancer, the benefit of modulating therapy during treatment to take advantage of evolutionary pressure and population dynamic to increase duration of response was demonstrated by Silva et al who described that the challenge of resistance and disease progression in metastatic breast cancer is the development of acquired resistance to chemotherapy by increased expression of the p-glycoprotein membrane pump that essentially removes chemotherapeutic agent from the cells. They demonstrated *in silico* and *in vitro* a prolonged time to resistance by combining standard chemotherapy, with dosing based on MTD and adaptive chemotherapy treatment with low dose verapamil and 2-deoxyglucose (172). Also in breast cancer, Enriquez-Navas et al. demonstrated a prolonged progression free survival (PFS) by modulating the dose of paclitaxel based on response to therapy *in vivo* (173).

This approach is also gaining traction in cancers that are treated with targeted therapy. In colon cancer, several studies have been implemented which take advantage of real-time assays of clonal predominance. Specifically, the CHRONOS (NCT03227926), RASINTRO (NCT03259009) and CRICKET (NCT02296203) trials are ongoing with the goal of identifying patients whose tumors may be sensitive to anti-EGFR therapy based on clonality results from cfDNA assessment.

Other strategies for monitoring clonal evolution before resistance development include the ongoing clinical trials in renal cell carcinoma (NCT01158521) and NSCLC (NCT03088930, NCT03433469) in which a patient’s tumor can be evaluated before and after a limited period of targeted therapy. Though subsequent therapy is not modulated based on the results in these cases, these studies are critical to assist in building an encyclopedia of response to treatment by tumors and the dynamic composition of the tumor ecosystem.

Combination Therapies

The challenges of single modality therapies are apparent in that the richness of tumor heterogeneity (including the tumor cells, the immune and micro-environment and epigenetic factors) essentially primes the tumor to evolve into a more molecularly complex and treatment-refractory organism. In NSCLC, activating mutations in EGFR predict for response to EGFR TKI treatment but most responses are incomplete and virtually all patients develop resistance that is mediated by multiple mechanisms (38,174–183). Jonsson, Blakely et al. highlighted the challenges of using targeted therapy in a heterogeneous tumor genomic environment given that the while targeted therapy is effective on the targeted clone, subclones are typically present that are less sensitive to the therapy. These subclones thrive in response to the selective pressure of a single-agent targeted therapy. This work demonstrated *in silico* and *in vitro* that utilizing dynamic treatment modulation both in response to changing subclone populations and in anticipation of subclone emergence, rational combination therapies can prolong the time to disease progression and in some cases via a non-intuitive switch therapy approach (184).

Other groups have demonstrated the benefit of combining targeted therapy with chemotherapy, integrating targeted therapy with antiangiogenic therapy and combination targeted therapies with improvements in PFS and OS. In the first randomized study to evaluate the benefit of combining an EGFR TKI with chemotherapy in patients with EGFR activating mutations, Sugawara et al evaluated the benefit of concurrent or sequential alternating gefitinib and chemotherapy in untreated NSCLC patients (NEJ005) (185). In 80 patients, the authors reported a median PFS of 18.3 months for concurrent treatment and 15.3 months and OS of 41.9 and 30.7 months, for the concurrent versus sequential alternating arms, respectively (185). In a follow up study, (NEJ009) the authors combined carboplatin/pemetrexed with gefitinib and demonstrated a superior OS compared with gefitinib monotherapy (52.2 months versus 38.8 months) (186). Similarly, in FASTACT-2 patients received doublet chemotherapy with either placebo or erlotinib on days 15–28 of up to six 28 day cycles followed by maintenance TKI. The PFS was 16.8 months in the chemotherapy with erlotinib group, compared with 6.9 months in the chemotherapy plus placebo group (HR 0.25 95% CI 0.16–0.39; $p < 0.001$). The median OS was 31.4 months in the combination group and 20.6 months in the chemotherapy with placebo group despite 85% of the placebo group patients going on to receive an EGFR TKI as subsequent treatment (HR 0.48 95%CI 0.27–0.84; $p = 0.0092$) (187).

In BRAF V600E melanoma and NSCLC, the combination of the two different targeted agents resulted in significant improvement in PFS and OS (188,189). The success of this combination is a testament to the benefit of combination therapy. Notably the lack of efficacy of dabrafenib and trametinib in the second-line setting after a BRAF-directed monotherapy highlights that tumors can become more complex under the pressure of therapy (190). These examples demonstrate the value of rational combination therapies given the superior OS noted in the experimental arms compared with standard sequential therapy.

A significant challenge in identifying effective combination therapies is related to the traditional clinical trial structure in that drug combinations need to be proven effective in late

stage disease before advancing to the frontline setting. As we have described above, cancers evolve continuously therefore, we risk overlooking effective combination treatments by requiring their validation in heavily pre-treated patients.

The idea of switching or intercalating two therapies with different mechanisms of action is not unique to cancer. This approach has been successfully employed in infectious disease for treatment of HIV, malaria and tuberculosis with success (191). As in infectious disease, it is critical to explore the benefit of radical treatment strategies at treatment initiation given that the more selective pressure cells undergo, the more genetically heterogeneous they become and ultimately, the more challenging the cancer will be to treat (38).

Longitudinal Monitoring of Evolution

Technical advances have revealed new ways to monitor treatment response and evolution of treatment resistance longitudinally. Serial biopsies at times of progression are important to monitor the somatic genomic and cellular changes as tumors evolve during treatment. Multiple repeat biopsies are not technically and logistically feasible in all patients and risk procedural complications. Furthermore, tissue biopsies can be molecularly biased given that they only reveal the tumor, immune and microenvironmental features of the individual sample, which may not be representative of the whole disease (3). Autopsy surveys are also often used in patients and provide invaluable information regarding tumor evolution and clonal population across disease sites globally and allow for reconstruction of evolutionary histories using phylogenetic analysis (192). However, the obvious issue with autopsy analyses is that it is a terminal endpoint and active modulation of therapy based on the findings is not feasible. Plasma sampling to evaluate cfDNA provides a minimally invasive way to assay the heterogeneity of the entire tumor cell population. Development of longitudinal plasma-based evaluation and the use of safe and informative tumor biopsy based serial protocols permits a more immediate approach to effectively monitor tumor evolution in the clinic (193,194). Limitations of plasma-based studies include the requirement of a baseline level of cfDNA present for detection and the challenge of recognizing complex genomic features (195). Several clinical trials are ongoing across multiple cancer types to evaluate the use of cfDNA in patients (NCT02443948, NCT03691012, NCT02245100, NCT03637686, NCT02883517).

In the preclinical setting, after the recognition of the importance of monitoring mutations and heterogeneity longitudinally the focus has shifted to understanding the transcriptome and how it evolves in concert with the genome. Given that the TME, immune cell, and tumor cell changes can influence the transcriptome, it is challenging to decipher the contributions of each of these populations. Bioinformatics analyses on total RNA sequencing can help in separating tumor, stromal, and immune cells. It is still difficult to prove whether this truly represents the individual contributions of these cell types (196).

The development of single cell analyses has been instrumental in allowing more accurate characterization of tumor and their microenvironment (197–204). In addition to characterization, these studies have identified interesting disease features that are important when considering treatment. For example, in head and neck cancer Puram et al analyzed the

TME in primary and metastatic disease sites. They identified an EMT signature that was an independent predictor of more aggressive disease (202). In melanoma, Su et al, demonstrated the potential of incorporating single cell analysis into monitoring disease longitudinally by utilizing whole-transcriptome analysis and single cell proteomic profiling to investigate the responses of patient-derived *BRAF* mutation positive cell lines to BRAF inhibitor treatment (204). They utilized single cell protein signaling network analyses to demonstrate that adaptive resistance begins with activation of MEK and NF κ B early in treatment, which allows cancer cells to persist despite BRAF inhibitor treatment. They demonstrated that with directed combinatorial therapy designed to inhibit specific protein signaling pathways, resistance was delayed. Similarly, Blakely et al demonstrated that NF κ B is activated early in NSCLC treated with an EGFR TKI and that NF κ B inhibition can attenuate resistance to treatment (205). These findings echo the results of Wei et al., in which the authors also employed single cell barcoded chip analysis to demonstrate the timeline and mode of adaptive signaling changes seen in response to treatment of a patient derived glioblastoma cell model (206). This type of analysis allows for determination of the protein levels of specifically queried proteins as well as protein-protein interactions within an individual cell. These studies highlight the value of auditing the proteome given that adaptive changes occur on a more rapid timeline and often involve post-transcriptional controls. Wei et al and Su et al demonstrated that protein networks evolve when treatment is applied resulting in increased functional heterogeneity of protein levels (more dispersion of functional protein levels) as well as increased activation of cell signaling networks that can confer resistance to targeted treatment (206). Adaptive responses to immunotherapy were identified in melanoma in which micro-environmental cues can lead to translational reprogramming and increased tumorigenesis and resistance to and anti-PD-1 treatment by repression of MITF and increased AXL (207). Adaptive signaling networks were characterized in CAFs in addition. Proteomic profiling and an in-depth bioinformatics analysis of CAF versus normal fibroblasts in patients with esophageal adenocarcinoma led to the characterization of the proteomic differences and possible therapeutic targets in the TME if there is a commonly active protein network (208).

Engineering Principles to Control the Multifactorial Basis of Tumor Evolution

A critical next step is to integrate and demonstrate these findings in patients, their convergence or privatization across cancer types, and to manipulate the causative protein signaling pathways before resistance develops to delay evolution and resistance to therapy. It is critical to determine how resistance changes signaling pathways and whether additional resistance mechanisms develop by manipulating evolution. Detailed work on modeling adaptive therapy approaches to treatment highlights the potential and challenges of this strategy to develop clinical trials (209,210). The primary challenge is applying the adaptive therapy models to patients and identifying clinically feasible ways for tracking tumor, epigenetic, and TME changes longitudinally during treatment at shorter intervals than imaging-based and tissue biopsies allow. One mechanism to implement clinically is “liquid biopsies” of tumor and micro-environmental features. In patients with targetable alterations, response to therapy in this way can be monitored in this way; utilizing this technology to

guide therapeutic options has not been widely implemented. As described above clinical studies to explore this strategy are ongoing (211–215).

In addition to real-time monitoring, we should consider the potential benefits of alternative clinical trial designs including combinatorial trials in early stage disease and switch therapy trials. A critical component to switch therapy is the determination of the optimal time to switch treatment. In HIV, which is a corollary to our proposition, treatment changes are focused on maximizing time to response with combination therapies and therapy switching (216). This strategy is implemented to minimize the emergence and virulence of resistance. These methods rely on the determination of viral load and viral genotype to identify the optimal timing of switch therapy (216). Multiple groups have identified important axioms that need to be considered to implement this type of anticipatory, instead of reactive treatment strategy in cancer (184). Incorporation of “liquid biopsy”-based information shows promise in monitoring response and relapse to treatment in lung, gynecologic, colon and hematologic malignancies (211–215).

We propose an adaptive trial design in which patients are treated with therapy combinations with frequent molecular/heterogeneity re-assessment to determine if this approach can prolong treatment response by capitalizing on intra-tumoral fitness competition. Once therapy combinations are deemed safe they should be rapidly evaluated in the first-line treatment setting given that tumor heterogeneity increases during therapy and responses to treatment diminish after multiple lines of therapy (38).

An idealized concept of a clinical trial is depicted in Figure 3. Patient specimens undergo molecular profiling (DNA, RNA, epigenetic, proteomic) before treatment to determine their mutational liabilities. This assessment would also allow for comparison of individualized analyte measurements during treatment and would permit more timely and sensitive monitoring of tumor burden (217). Patients would first receive alternating targeted therapies in combination with an inhibitor of a known survival/bypass pathway, such as MAPK, AKT, or NF κ B signaling (205). Based on the profile changes identified after the initial combination therapy, patients would then continue their current regimen, or switch to an alternate therapy as informed by the residual disease profile that is detected by serial molecular profiling. This treatment would then be subject to change to an alternative regimen dictated by the continued evolution of the molecular profile as assessed at defined intervals with ctDNA and tumor biopsy re-assessment.

By utilizing alternating therapies in the initial treatment setting, tumor growth could be better managed as a chronic disease. The potential of this approach is described above in multiple myeloma, in which two competing sub-clones alternated dominance based on treatment until a separate dominant mechanism of survival evolved (71). The potential for this same approach is seen in NSCLC with EGFR activating mutations. In this setting, EGFR T790M positive tumor cells (first generation EGFR TKI resistant but third generation EGFR TKI sensitive clones) grow more slowly than EGFR T790M negative tumor cells (218). A potential strategy for improved control of tumor cell subpopulation dynamics is to engineer a mixed population of EGFR T790M positive and negative tumor cells that would compete for dominance during alternating treatments with first and third generation EGFR

TKIs. A visualization of this clonal interaction and competition is illustrated in Figure 4. To further complement the clonal competition, we have additionally proposed to include an inhibitor of a common bypass pathway likely to be active in both predominant subclonal populations.

A challenge of this and other adaptive approaches is the continual re-assessment of tumor biology. This is more feasible in certain patients and cancers than in others. In addition to improving clinical outcomes, this clinical trial design would help to develop an encyclopedia of treatment response and resistance and determination of the minimum input needed to make treatment decisions effectively. The complexity of evolution in the setting of tumor heterogeneity makes this approach challenging. Yet, “realtime” monitoring in an evolutionary trial design is critical in order to leverage biological insights into tumor evolution to engineer principled therapeutic strategies for improved, chronic control of cancer.

Summary

Tumor biology is a much more complex and integrative process than originally recognized. Through the development and evolution of technology we now more fully appreciate the diversity between cancer types, within cancer types, subtypes, microenvironment and cancer cells themselves. We have learned that resistance to treatment is a synthetic phenotype, which must be considered when devising treatment and strategies to attenuate resistance to therapy. We must focus on principled approaches to engineer evolution to control the process and thereby advance cancer patient survival.

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Statement of Significance

Tumor and tumor immune compartment and micro-environment heterogeneity, and their evolution are critical disease features that impact treatment response. The impact and interplay of these components during treatment are viable targets to improve clinical response. In this article, we consider how tumor cells, the tumor immune compartment and microenvironment, and epigenetic factors interact and also evolve during treatment. We evaluate the convergence of these factors and suggest innovative treatment concepts that leverage evolutionary relationships to limit tumor growth and drug resistance.

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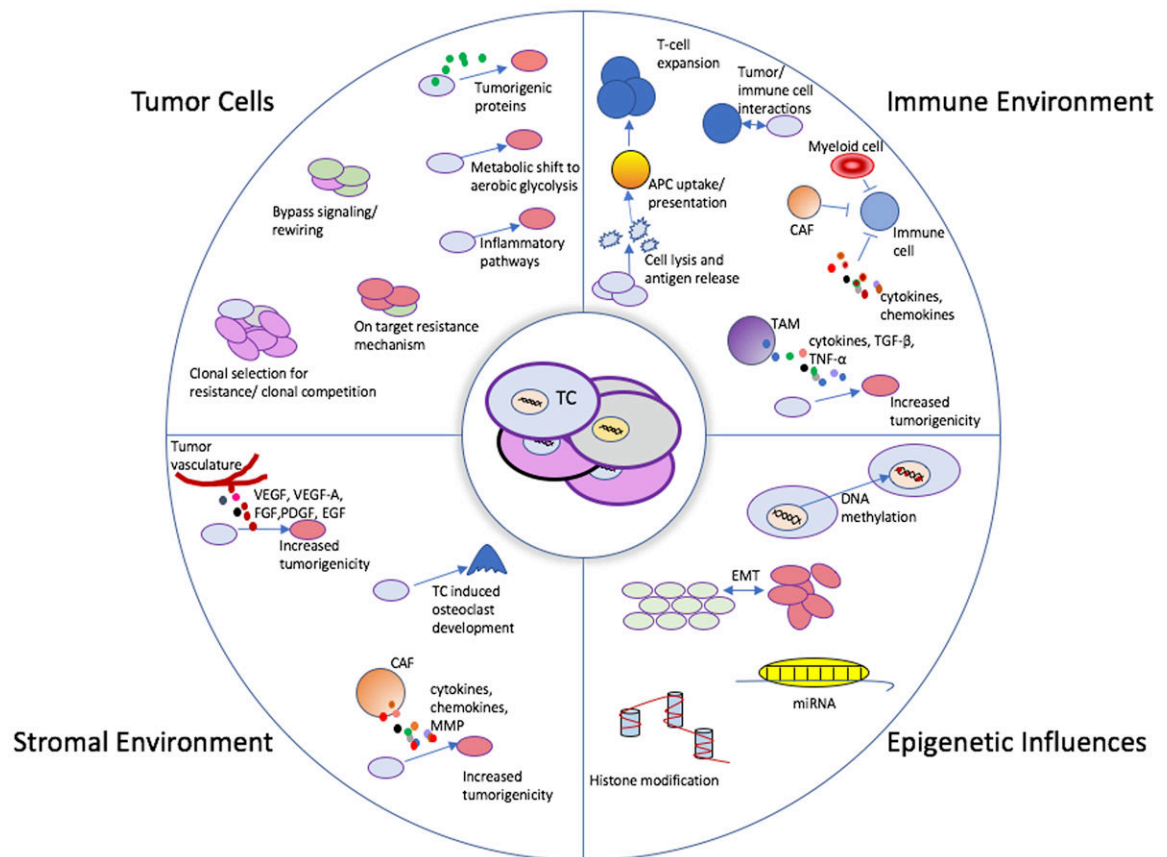


Figure 1. Tumor cell influences and evolution.

Depiction of the evolutionary paths of tumor cells. The center portion of this image depicts a group of heterogeneous tumor cells. There are four predominant, and often overlapping, categories of evolutionary influence that can impact tumor cells during cancer progression and treatment that impact response to therapies. Each category depicts several individual mechanisms of tumor promotion and evolution. These mechanisms are not mutually exclusive and can occur concurrently.

Abbreviations: APC- antigen present cell, CAF- Cancer associated Fibroblast, EGF – Endothelial growth factor, EMT – epithelial to mesenchymal transition, FGF – Fibroblast growth factor, miRNA- micro-RNA, MMP- Matrix metalloproteinases, PDGF – platelet derived growth factor, miRNA- micro-RNA, MMP- Matrix metalloproteinases, PDGF – platelet derived growth factor, TAM – Tumor associated macrophage, TC- tumor cell, TGF- β – transforming growth factor beta, TNF- α – tumor necrosis factor alpha, VEGF/ VEGF-A - vascular endothelial growth factor (A),

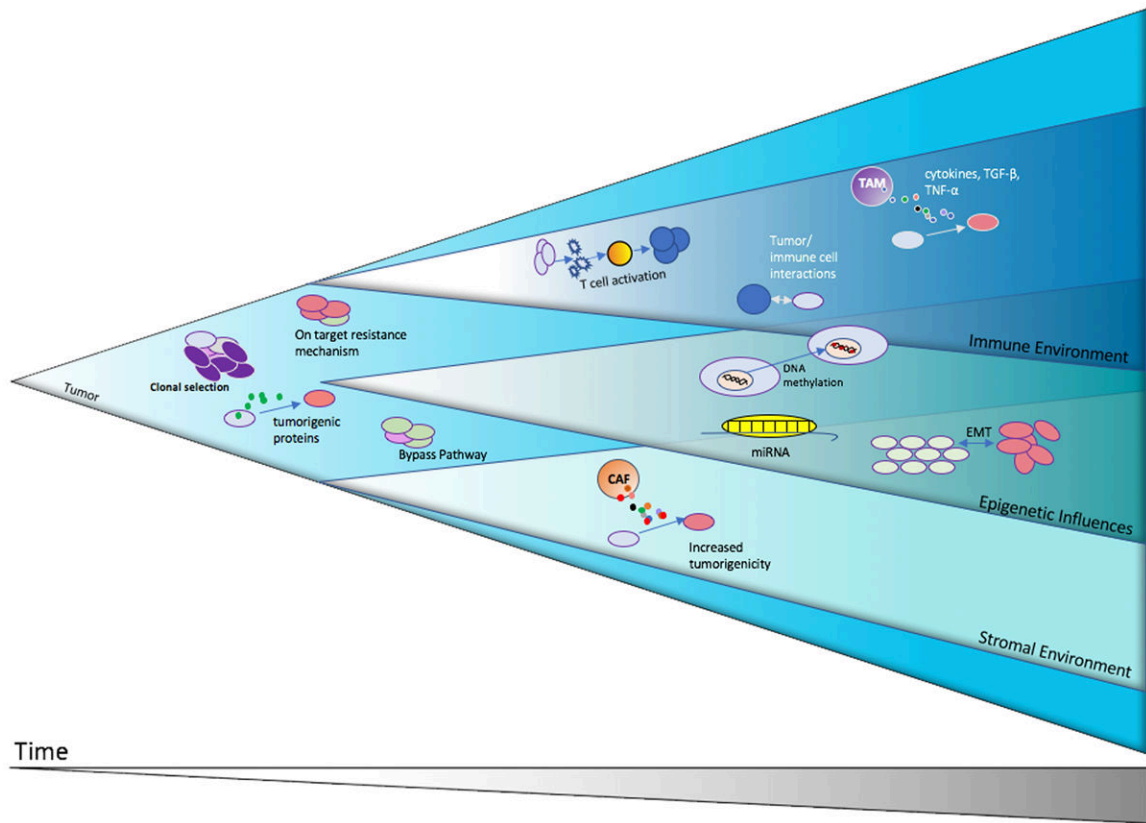


Figure 2. Depiction of tumor evolution.

Shown is a theoretical illustration of tumor growth and evolution demonstrating how some of the different modes of tumor promotion and evolution in response to therapy may occur and evolve concurrently.

Abbreviations: CAF- Cancer associated Fibroblast, EMT – epithelial to mesenchymal transition, miRNA- micro-RNA, TAM – Tumor associated macrophage, TGF- β – transforming growth factor beta, TNF- α – tumor necrosis factor alpha

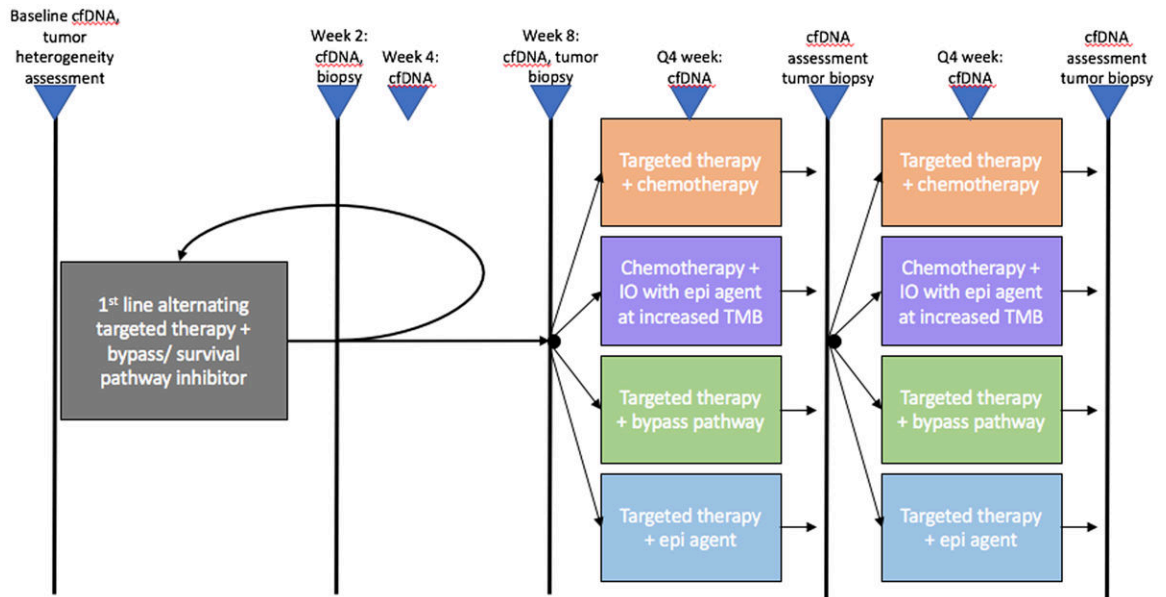


Figure 3. Biological and learning based molecular treatment of cancer.

Depiction of a clinical trial design in which tumors and cfDNA are evaluated longitudinally during treatment to tailored therapy combinations based on the evolution of the persistent, or emergent clonal populations identified during active treatment and before treatment resistance is detected using more conventional clinical or radiographic assessments.

Abbreviations: cfDNA – cell-free DNA, IO – immunotherapy, TMB – tumor mutation burden, epi – epigenetically targeted agent.

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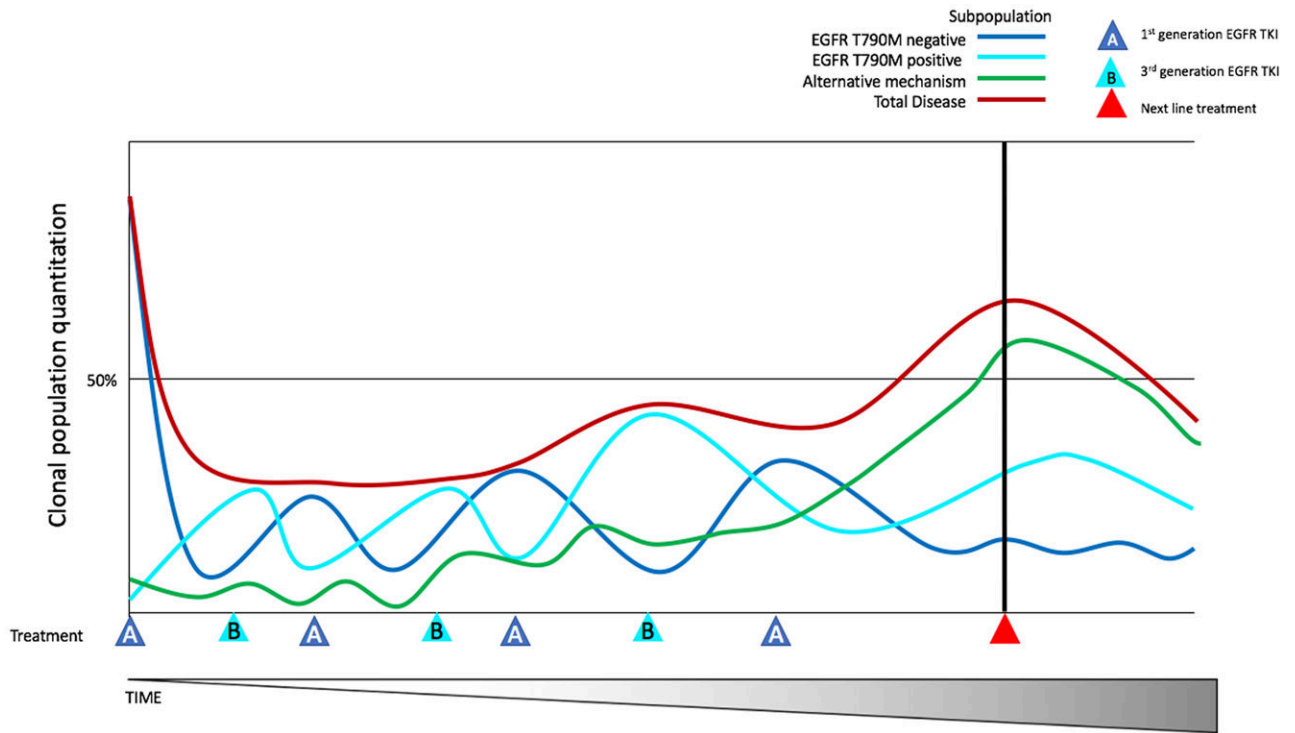


Figure 4. Clonal predominance in alternating therapy

Depiction of clonal predominance switching during alternating treatment with 2 different EGFR TKI therapies to maintain a steady state population until outgrowth of a clonal population resistant to both therapies occurs, at which point a new therapy would be employed.