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Intertumoral and Intratumoral Heterogeneity of Cancer: Types, Causes, Detection, and Clinical Implications

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Abstract

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By Rohini Neha Guin

Intertumoral and intratumoral heterogeneity of cancers pose significant challenges to the development of effective antineoplastic therapies. Tumors contain a complex admixture of different clonal populations, each of which may vary in their mechanisms of proliferation and maintenance. The latest technology developments in the genomic and proteomic areas further define tumor heterogeneity at the molecular level, making it feasible to develop personalized treatment approaches. This review will highlight the types of heterogeneity, the ways in which heterogeneity arises, and how heterogeneity can be evaluated. Further, this review will explore current and emerging immunotherapies and personalized treatment strategies to best overcome tumor heterogeneity.

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Introduction

Cancer refers to a group of heterogeneous diseases that can originate from multiple cell types. Cancer cells are distinguished from normal cells through the display of the following hallmarks: uncontrolled growth, resisting cell death, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg, 2011). The intrinsic and extrinsic factors leading to these hallmarks vary among individuals, resulting in genetic mutations that vary from person-to-person.

Recent advances in sequencing and bioinformatics have been instrumental in revealing the genetic diversity of cancers (Andor et al., 2012, Beck, 2015, Jiang et al., 2016). Genomic studies have also uncovered the complex clonal landscape of tumors (Shah et al., 2012, Siravegna et al., 2015). The observed variation across cancer cell populations is called tumor heterogeneity. These clones may also differ across patient populations and within the same patient, resulting in intertumoral heterogeneity and intratumoral heterogeneity, respectively.

The dynamic evolution of cancer cells can cause cells of one bulk tumor mass to harbor distinct molecular signatures and differential sensitivities to antineoplastic therapies. Highlighting these tumor heterogeneities is a central goal of this review. We also aim to address the factors that underlie the acquisition and maintenance of intertumoral and intratumoral heterogeneity. Further, we summarize methods for quantifying and evaluating heterogeneity, in addition to methods for diagnosing and treating a particular subtype of cancer. Finally, we highlight various therapeutic approaches employed to overcome tumor heterogeneity, a considerable barrier to anticancer treatment development.

I. Types of Cancer Heterogeneity

Inter- and intra- tumoral heterogeneity operate on multiple levels. Observed molecular heterogeneity of cancer cells can be attributed to differences in genetic, transcriptional, epigenetic, microenvironmental, structural/spatial, and macroenvironmental states, as discussed below (Fig. 1). Understanding the types of heterogeneity markers and mechanisms can aid the development of antineoplastic therapies.

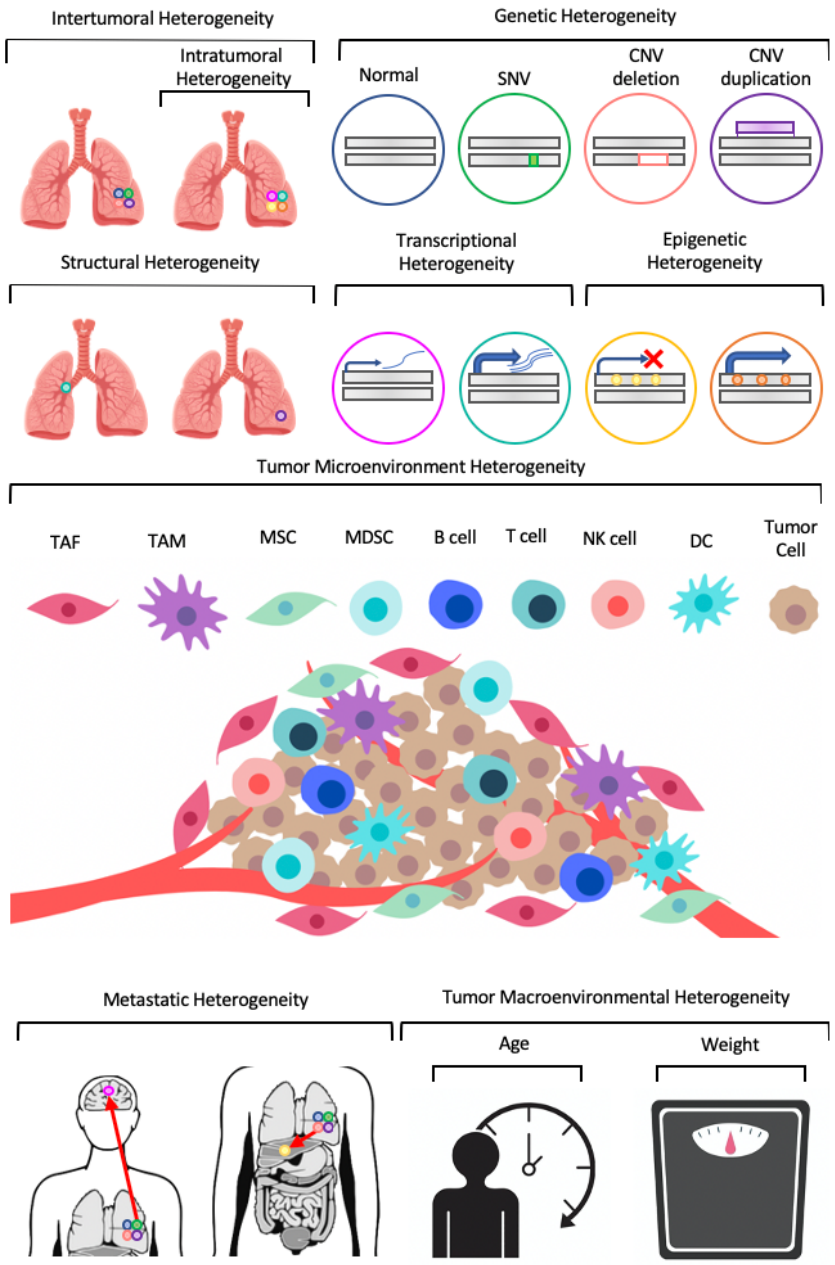

Figure 1. The different levels of tumor heterogeneity. Using a lung cancer example, intratumoral heterogeneity arises from the various clones within one patient, whereas intertumoral represents differences in clones across patients. Genetic heterogeneity demonstrates chromosomes may acquire a single nucleotide variant (SNV) or a copy number variant (CNV). The CNV can result in gene suppression when copy numbers are decreased and gene amplification when copy numbers are increased. Structural heterogeneity refers to the histological and spatial location of the tumor. In lung cancer, tumors can arise in the bronchi or the alveoli. Transcriptional heterogeneity is characterized by differences in gene expression, quantified by mRNA transcript levels. Epigenetic heterogeneity is marked by DNA or histone modifications that may alter gene expression profiles. The tumor microenvironment shows cancer-associated fibroblasts (CAF), tumor-associated macrophages (TAMs), mesenchymal stem cells (MSC), myeloid-derived suppressor cells (MDSC), B cells, T cells, natural killer (NK) cells, and dendritic cells (DC) surrounding a tumor cell population. Blood vessels are depicted by red branches. Metastatic heterogeneity refers to differences in metastases from primary tumor or between sites of metastasis. The tumor macroenvironment shows factors like age and weight that are relevant to physicians selecting treatments for their patients.

Genetic Heterogeneity

Genetic heterogeneity refers to the genetic alterations harbored by cancer cell populations. These genetic alterations may be single nucleotide variants (SNVs), such as single base substitutions (missense, nonsense, and mutations in untranslated regions) and frameshift mutations brought about by single nucleotide insertions or deletions. SNVs lead to drug resistance and have clear clinical implications on the course of treatment, thus identifying common mutants may provide new targets for drug treatment. The misregulation of the DNA deaminases APOBEC

family of enzymes can give rise to SNVs. APOBEC3B is upregulated along with its target sequence in bladder, cervix, lung adenocarcinoma, lung squamous cell carcinoma, head and neck, and breast cancer (Burns et al., 2013). SNVs also account for inter- and intratumoral heterogeneity across several cancers and their subtypes. One study evaluating heterogeneity in synchronous colorectal cancer (sCRC, colorectal cancer with more than one primary tumor), found that only 3 of 23 patient cases consisted of genetically identical tumors; the remaining 20 cases (87%) showed intertumoral heterogeneity (Jesninghaus et al., 2016). Intratumoral evaluations of multiple primary tumors within the same patient showed that 44% of patients harbored one KRAS wildtype tumor and another KRAS mutant tumor (Jesninghaus et al., 2016). 48% of the cases also had double, triple, or quadruple mutations in KRAS, APC, TP5, PIK3CA, and TGFBR2 (Jesinghaus et al., 2016). This study highlights the mutated genes most implicated in the development of sCRC, in addition to the variation found across patients and within patients. A study of esophageal cancer using whole exome sequencing identified 90% intratumoral heterogeneity with SNVs in microRNA-binding sites MHFR, MYH11, ABCC4, GRPC6A, TP53, HGF, and PIK3R2 genes. Various somatic SNVs are implicated in a range of cancers.

Another type of genetic aberration includes copy number variants (CNVs), which are alterations resulting in increased or decreased numbers of DNA segments >1,000 bases in length (Shao et al., 2019). These anomalies can be just as destabilizing as SNVs. One study finds CNV burden of 50-75% in the tumor genome corresponds to higher mortality risk in comparison to CNV burdens <25% and >75% (Andor et al., 2012). Another study identified CNVs through array-based comparative genomic hybridization in two patients with esophageal squamous cell carcinoma; the results identified 430 and 262 amplification or deletions in each, resulting in the amplification of PRKCI, SOX2, and CCTN (Cao et al., 2015).

When considering both SNVs and CNVs, the concept of a clone also arises. A clone is defined as a tumor population with similar mutation profiles, while subclones are cell populations that diverge from the shared traits common to the original clone. A pan-cancer study used tumor separation algorithms PyClone and EXPANDS to evaluate intratumoral heterogeneity in 12 cancers (thyroid, prostate, kidney, low-grade glioma, glioblastoma, head and neck, cervical, stomach, lung (adenoma), lung (squamous), melanoma, and bladder). The study revealed that more clonal diversity does not linearly correlate with poor cancer prognosis (Andor et al., 2012). Rather, 1-2 clones and >4 clones result in improved prognostic outcomes, especially when there are more CNVs (Andor et al., 2012). These findings point to an optimal number of clones harbored in tumors. This knowledge can help personalize treatment based on the number of clones. Patients with 3-4 clones may benefit from DNA-destabilizing treatments, like radiation and chemotherapy, to increase the number of clones. Patients with 1-2 clones may benefit from more targeted treatment approaches.

One of the limitations of these findings is introduced from the Cancer Genome Atlas (TCGA) dataset, which is a large scale genome profiling effort that details primary tumors genetic data, but excludes data on xenografts, cell lines, and has limited data on metastases; all these additional sources of genetic data could help identify a more representative range of SNVs and CNVs (Liu et al., 2018, Solomon et al., 2009). Canopy, another statistical framework used to develop tumor phylogenies, has been tested in four datasets, including the MDA-MB-231 breast cancer cell line, breast cancer xenografts, leukemia, and ovarian cancer. This framework has also been a powerful tool in tracing tumor heterogeneity of clones overtime (Jiang et al., 2016). The test samples used in Canopy differ drastically from the TCGA dataset, so it is important to evaluate these SNVs and CNVs using both datasets to ensure unbiased findings.

Driver mutations are another class of mutations, attributed to the acquisition of cancer-like phenotypes in tumor cells. This differs from passenger mutations, which are secondary mutations that do not contribute to tumorigenesis. In a pan-cancer evaluation of sixteen different cancers, researchers found 66% of cancer-driving mutations are due to errors in DNA replication, while only 29% and 5% of mutants arise from environmental factors and inherited mutations, respectively (Ledford, 2017). Other studies place less emphasis on the significance of cancer driver mutations. For example, one study finds that 12% of triple-negative breast cancers contained no genetic anomalies in known drivers (TP53, PIK3CA, and PTEN to name a few) or cytoskeletal genes (Shah et al., 2012). This finding implies that other unknown anomalies may be attributed to driver-free TNBCs. The finding also raises the question of what drives early clonal expansion in these cancers.

Beyond questions raised about the origins of cancer, it is generally agreed that genetic mutations can contribute to the development of cancer. Others point out that it is difficult to filter tumor drivers from passengers, given that the roles of driver and passenger mutations may switch as tumors evolve (Polyak, 2011). Further, some studies caution against overlooking passenger mutations, since their accumulation can collectively have a deleterious effect and result in cancer meltdown (McFardland et al., 2014). In a model of over 2,500 cancer genomes, the aggregate effect of putative passengers provided significant additional power for predicting cancerous phenotypes, and passengers in tumor suppressors may confer weak driver activity (Kumar et al., 2020). Another study evaluating multiple driver mutations in 60,954 cancer samples, found six main clusters. Cancers with driver mutations in PIK3CA and NOTCH were more susceptible to inhibitory drugs in comparison to cancers with either singular driver mutation (Saito et al., 2020).

Nevertheless, the driver and passenger mutation distinction is still significant, as it aided in the identification of key genes contributing to tumorigenesis.

Transcriptional Heterogeneity

In addition to variations in the genetic code, gene expression is just as critical to defining tumor prognosis. Even within isogenic cell populations, variation in gene expression can determine why certain subsets of the population die when exposed to drug agents while others survive (Paek et al., 2016). Additionally, several mutations found in cancers may not be expressed and will therefore not actively contribute to the disease phenotype. One high throughput RNA-sequencing analysis of TNBCs shows that only 36% of all mutations in the genome were expressed (Shah et al., 2012). Studies like these show that analysis of the transcriptome is also relevant to evaluating tumor heterogeneity.

Transcriptional heterogeneity has been identified in several instances of tumor heterogeneity. Microphthalmia-associated transcription factor (MITF) is associated with increased cell differentiation in melanoma (Shannan et al., 2016).In a metastatic melanoma study, bulk tumors were evaluated with whole exome sequencing and single-cells were analyzed by RNA-seq. The study identified two transcriptional states in pretreated melanoma subpopulations that corresponded to either a high expression of the MITF gene (or other downstream targets which was grouped into a ‘MITF-high’ designation) or a low expression of MITF and high AXL. On a single cell level, however, there was a differential level of MITF expression, even when the bulk tumor was easily categorized with a single expression profile (Tirosh et al. 2016). These findings highlight how inter- and intra- tumoral heterogeneity can occur on the transcriptional level.

Transcriptional heterogeneity can be mediated by epigenetic modifications. The same melanoma study also reveals heterogeneity in cell cycle gene expression, where non-cycling cells have high levels of KDM5B (Lysine Demethylase 5B) expression relative to cycling cells (Tirosh et al., 2016). These non-dividing cells are not sensitive to cell-cycle targeting drugs and tend to persist in patients even when dividing cancer cell populations are cleared, pointing to the need of combination treatments that can act on this resistant population. In another study, increased KDM5B activity resulted in increased transcriptomic heterogeneity, leading to endocrine therapy resistance in ER^+ breast cancers (Hinohara et al., 2018). In patients demonstrating this transcriptomic heterogeneity, introducing an epigenetic targeting agent may improve efficacy and overall sensitization. Gene expression of housekeeping genes and transcription factors are also differentially expressed across cancers. In a sample of 567 ER^+ breast cancer, simultaneously elevated MYC and RAS activity confer a significantly worse prognosis than either high MYC or high RAS activity alone (Teschendorff et al., 2010). In a molecular analysis of small cell lung carcinoma (SCLC) in genetically engineered mouse models (GEMMs), double knockouts (KOs) of tumor suppressor genes RB1 and TP53 gave rise to differential gene expression patterns, including one transcriptional state of ASCL1-high and NE-high and one transcriptional state of ASCL-low and NE-low, while triple KOs of RB1, TP53, and RBL2 demonstrated variable expression of NFIB in metastases (Pozo et al., 2018). The loss of RB1 may contribute to increased neuroendocrine cell plasticity and differentiation into new populations (Yang et al., 2018). Despite having the same lineage and histology of cells, variable gene expression patterns result in differential outcomes.

Epigenetic Heterogeneity

The modulation of chromatin structure through the modifications of histones or DNA can result in varied gene expression patterns across patients. While transcriptomic heterogeneity may arise from epigenetic changes, increased gene expression can also be attributed to variation in copy numbers of genes. For example, in a hepatocellular carcinoma model, single-cell triple-omics sequencing method of the genome, methylome, and transcriptome revealed CNVs are proportional to RNA expression, but do not impact the DNA methylation of these regions (Hou et al., 2016). Epigenetic modifications are simply one facet that can contribute to transcriptional heterogeneity.

There are two main classes of epigenetic aberrations implicated in cancers. The first class of epigenetic changes are due to somatic mutation in an epigenetic regulatory protein, resulting in aberrant gene expression of targets. Drug-resistant or drug-tolerant cell states may be transient and reversible if mediated by epigenetic modification, increasing interest in the development of inhibitors against proteins modulating gene expression (Sharma et al., 2010). One target of interest is KDM5B, a gene that codes for a histone H3 lysine 4 (H3K4) demethylase which can increase gene expression. Estrogen receptor-positive (ER^+) breast cancers can be sensitized to anti-estrogen drugs by knocking out or inhibiting KDM5B, since high KDM5B expression results in higher transcriptomic heterogeneity and more cancer clones (Hinohara et al., 2018). KDM5A has also been implicated in drug resistance in lung cancers (Sharma et al., 2010). These findings illustrate the need for epigenetic targeting agents to decrease tumor progression and supplement other standard-of-care treatments. Another epigenetic regulator implicated in cancer development is DNA methyltransferase 3A (DNMT3A). Somatic mutations in DNMT3A were identified in acute myeloid leukemia M5 subtype in 23 of 112 cases (20.5%) (Yan et al., 2011). The aberrant activity of mutant DNMT3A resulted in variable expression of HOXB (Yan et al., 2011). HOXB genes are

transcription factors that target gene regulating protein sequences, so aberrant HOXB activity can lead to further abnormalities in gene expression.

The second major class of epigenetic alterations are modifications of promoters of gene families (such as DNA repair proteins), which result in the accumulation of genetic mutations that increase cancer risk. Cytosine-phosphate-guanine-rich regions of DNA, termed CpG islands, are often sites of hypermethylation across various cancer types. Cytosine can spontaneously be deaminated, resembling uracil, a RNA base. However, CpG islands contain several 5'-methylcytosines, which, when deaminated, produce a thymine base. Correcting a C>T substitution is more difficult than a C>U, especially when DNA mismatch repair (MMR) proteins are insufficiently active. In a study evaluating sCRC, the CpG island of MLH1 was found to be hypermethylated in several cases and increased hypermethylation correlated to increased BRAF V600E mutants (Jesinghaus et al., 2016). CpG islands have also been differentially methylated in cancer subtypes. Hypermethylation within ESR1-containing CpG islands was found in ER^- breast cancers, but not in ER^+ breast cancers (Bertos and Park, 2011). CpG island hypermethylation results in the inactivation of PTEN (25%) and CDKN2A (50%), two common driver mutations of melanoma (Shannan et al., 2016). CpG island hypermethylation events are clearly implicated across cancer types, and when particular regulatory genes are silenced, somatic mutations can accumulate and increase cancer risk. Thymine DNA glycosylase (TDG), an enzyme involved in base excision repair, is hypermethylated in the promoter region of several multiple myeloma cell lines, resulting in lower gene repair of hydrogen peroxide-induced damage (Peng et al., 2006). Another gene, 8-oxoguanine DNA glycosylase (OGG1), is hypermethylated across breast cancer types and results in less oxidation repair to guanine bases, which can result in the accumulation of deleterious mutations (Fleischer et al., 2014). Additionally, hypomethylation of satellite 2

repetitive element (SAT2), ALU, and long-interspersed nuclear element-1 (LINE-1) correlates with increased cancer incidence (Guo et al., 2019). These types of epigenetic abnormalities are more difficult to target since the cause of hypermethylation or hypomethylation remains unknown. Still, in cases of hypermethylation and reduced transcription of DNA regulatory elements, the findings may be relevant to the development of treatments where those proteins are exogenously introduced to cells.

Cellular Tumor Microenvironment Heterogeneity

The tumor microenvironment (TME) plays a critical role in regulating tumorigenesis. Cancer proliferation depends on the interaction of tumors with the noncancerous cellular landscape. These cellular components include cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), mesenchymal stem cells (MSCs), myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), natural killer (NK) cells, B cells, and T cells. The TME is capable of changing the tumors and cancers are capable of manipulating the TME (Jin and Jin, 2020).

a. Cancer-Associated Fibroblasts

Fibroblasts are a cellular stromal component involved in tissue restructuring. Fibroblasts can be activated in wound-healing events in a similar fashion to CAFs found near tumors, which were characterized as “wounds that do not heal” (Dvorak, 1986). CAFs tend to have pro-tumor effects, but there are no well-defined molecular markers of CAFs that distinguish them from normal fibroblasts (Öhlund et al., 2014). CAFs are defined on a location-basis as the collection of fibroblasts found in a tumor. The variation in the molecular markers expressed of CAFs could be attributed to differences in embryogenic origins of non-cancerous fibroblasts from both the mesenchyme (Haniffa et al., 2009) and the neural crest (Sharpe, 2001), components of the

mesoderm and ectoderm, respectively. Tissue-resident fibroblasts also vary considerably and can account for the variance in molecular heterogeneity. Growth factors such as TGF-β have led to increased activation of CAFs via epigenetic modifications (Hu et al., 2005). CAFs were found in higher levels in recurrent melanoma cells which expressed high levels of transcription factor AXL (Tirosh et al., 2016). In breast cancer stem cells, CAFs have been shown to secrete IL-6 and IL-8, which activate the STAT3/NF-κB pathway and generate a positive feedback loop (Korkaya et al., 2011). In colorectal cancers, CAFs mediate cellular cross-talk with tumor populations through the release of miRNA-containing exosomes (Bhome et al., 2017). In addition to interaction with the tumor cell population, CAFs tend to have immunosuppressive effects. In human pancreatic cancer surgical resections, CAFs expressed higher levels of PD-L1 and PD-L2 in comparison to healthy donors (Gorchs et al., 2019). CAFs were demonstrated to increase T cell expression of exhaustion markers such as TIM-3, PD-1, CTLA-4, and LAG-3 (Gorchs et al., 2019). In human non-small cell lung cancer (NSCLC), CAFs derived from the primary tumor demonstrated strong immunosuppressive effect on activated T cells, even after high dose (18 Gy) radiation therapy, and the irradiated CAFs continued to release such as PGE_2, IL-6, IL-10, and TGF-β at comparable levels to non-irradiated CAFs (Gorchs et al., 2015). It is still unclear if CAFs are in a transient state or are in a committed lineage of pro-tumor cells. However, if treatments targeting the production of pro-tumor proteins like TGF-β are used, the tumors may be further sensitized to other therapies like PD-L1 blockade (Mariathasan et al., 2018).

b. Tumor-Associated Macrophages

Macrophages are a component of the innate immune system that are largely responsible for engulfing and degrading pathogens and cancer cells but degrade cellular debris in homeostatic conditions. They are generally thought to be derived from monocytes, although some tissue-

resident macrophages, such as those in the brain (microglia) and liver (Kupffer cells), have less clearly defined origins (Varol et al., 2015).

There are two main subpopulations of macrophages. These include classically activated M1 macrophages, which have a proinflammatory role, and alternatively activated M2 macrophages, which are involved in tissue remodeling and angiogenesis. TAMs are defined by the pathology they are associated with, namely cancer. Although TAMs share more similarity to M2 macrophages, they also possess M1 signatures and should be considered a separate grouping (Chávez-Galán et. al, 2015). Macrophages demonstrate tumor-promoting activity that can be diminished through monoclonal antibodies targeting pro-tumor cytokines released by TAMs and abolishing the interaction between TAM receptors and their ligands on tumor cells. One such interaction of interest is between CD47 ligand, a potent "don't eat me signal" expressed on tumor cells, and the SIRP-α receptor found on macrophages. In human non-Hodgkin lymphoma-engrafted mouse models, treatment with anti-CD47 antibody in addition to rituximab (anti-CD20 antibody) showed complete tumor clearance and enabled phagocytosis of the tumor by macrophages (Chao et al. 2010a). Another study by the same research group found calreticulin is a marker recognized by macrophage to induce phagocytosis, and tumor cells (acute myeloid and lymphoblastic leukemias, chronic myeloid leukemia, non-Hodgkin lymphoma (NHL), bladder cancer, glioblastoma, and ovarian cancer) often co-express CD47 and calreticulin to evade immune surveillance (Chao et al., 2010b). Calreticulin is minimally expressed on normal cells, indicating that an anti-CD47 antibody may be a good treatment option to maximize the "eat me" signal of calreticulin found on cancer cells. In addition, TAMs secrete IL-6, which activates the STAT3/NF-κB pathway in breast cancers (Korkaya et al., 2011). Another group found $CD68^{+}$ macrophages can secrete IL-6, which can increase the activation of the STAT3/ERK and Wnt/β-catenin

pathways, mediate the epithelial-to-mesenchymal transition (EMT) via the upregulation of transcription factor Snail, and promote increased migration and invasion of SW48 colon cancer cells (Gao et al., 2018). They also tested a JAK inhibitor, AG490, which was capable of reversing the STAT3/ERK activation pathway and decreasing the effect of IL-6 secretion from the TAMs (Gao et al., 2018). TAMs have also been implicated to increase the proliferation of gastric cancer through exosomes rich in ApoE, which can activate the PI3K pathway in tumor tissue (Zheng et al., 2018). Targeting pro-tumor TAM activity can help elicit tumor-suppressive responses to cancer.

c. Mesenchymal Stem Cells

MSCs are a stromal cell component isolated from the bone marrow and capable of differentiating into mesenchymal tissues such as bone, cartilage, and fat. MSCs may also be a source of CAFs (Trivanović et al., 2016). MSCs demonstrate dual immunomodulatory effects. MSCs are involved in structuring tissue via the production of extracellular matrix components, such as collagen, fibronectin, laminin, heparan sulphate, and proteoglycans. These modifications may facilitate tumor metastasis or help to contain tumors (Poggi et al., 2014). MSCs can also suppress immune function through a number of factors, like IDO, heme oxygenase (HO), arginase -1 and -2, nitric oxidase synthase 2 (NOS_2), HGP, TGF-β, IL-10, PGE_2, and HLAG (Poggi et al., 2014). MSCs can also help promote angiogenesis through the release of different pro-tumor factors. Breast cancer cells are found to stimulate CCL5 secretion of MSCs, which can enhance angiogenesis through paracrine signaling of CCR5 expressed on tumor cells (Karnoub et al., 2007). In ovarian cancer, carcinoma-MSCs enhanced tumorigenesis through increased BMP2, BMP4, and BMP6 expression (McLean et al., 2011). MSCs fueled cancer stem cells in breast cancers

through CXCL7 secretion (Korkaya et al., 2011). The release of these different factors and their receptors could be targeted by antibody therapy to prevent tumor progression.

d. Myeloid-Derived Suppressor Cells

MDSCs are a heterogeneous cell population that expand in cancers and can suppress T cell responses. They generally have pro-tumor effects, but have demonstrated some anti-tumor activity, as seen through their modulation of NK cells. For example, Gr-1$^+$CD11b$^+$F4/80$^+$ MDSCs isolated from RMA-S tumor-bearing mice do not suppress NK cells, but instead activate them to produce high amounts of IFN-γ through RAE-1, the activating signal of NKG2D receptor (Nausch et al., 2008). However, in hepatic cancer, CD11b$^+$Gr-1$^+$ MDSCs were found to expand during tumor progression, suppressing NK cell cytotoxicity, NKG2D expression, and IFN-γ production via TGF-β1 production (Li et al., 2009). Another study of T cells highlights MDSC dual function. A study using in vitro coculture systems showed that splenic MDSCs can suppress CD8$^+$ T cell proliferation but are mediated by different factors like IFN-γ, STAT-1, IRF-1, and NO (Schouppe et al., 2013). Further, both monocytic and granulocytic MDSCs stimulate CD8$^+$ production of IFN-γ, demonstrating T cell anti-tumor activity (Schouppe et al., 2013). Thus, MDSCs can have immunosuppressive and immunogenic effects mediated by direct contact with other immune cells.

e. Dendritic Cells

DCs are antigen-presenting cells that play a key role in immune-induction through direct activation of lymphocytes. DCs can also be polarized towards a pro-tumor and immunosuppressive state. Tumor-infiltrating DCs have been implicated in clinical outcomes of various cancers. If DCs are polarized towards an immunosuppressive-dominant state, they may promote a more regulatory response in T cells, thereby increasing tumor promotion. Tumor infiltrating DC precursors are also involved in tumor vasculogenesis; tumors recruit these DCs with β-defensins and use VEGF-A to

induce endothelial-like migration to vessels (Conejo-Garcia et al., 2004). DCs are also able to produce IL-12 and Type I IFN, two pro-inflammatory markers that can induce immunogenicity (Chow et al., 2012). Characterizing the signaling factors that promote specific DC states is important in the development of immunogenic DC-based cancer vaccines.

f. Natural Killer Cells

NK cells are lymphoid derivatives that can target cancers through the secretion of cytolytic granules, prompting an immunogenic response. Unlike B and T cells, which are also lymphocytes, NK cells do not display antigen specificity and mainly attack tumors which do not express MHC Class I/ due to loss of β2-microglobulin (β2M) (via loss of heterozygosity or point mutations) (Sade-Feldman et al., 2017). NK cells can be useful in targeting melanoma cancers that have downregulated β2-microglobulin and MHC Class I presentation (Porgador et al., 1997, Sade-Feldman et al., 2017). Damaged DNA can stimulate the induction of NK cells by activating signals such as NKG2D (Chow, 2012). However, tumors can evolve to evade immune surveillance through various mechanisms, such as ligand shedding. One such mechanism finds tumors that release soluble ligands of NKG2D have decreased interactions with NK cells and T cells (Iannello, 2016). Another team found that $CD4^{+}CD25^{+}$ regulatory T cells (Tregs) are able to suppress NK activation through the release of TGF-β, which inhibits the release of NKG2D receptors on NK cells (Ghiringhelli et al., 2005). In mouse models with depleted Tregs, there was a notable increase in NK cell proliferation and cytotoxicity (Ghiringhelli et al., 2005). NK cells can also have an immunosuppressive effect on other immune cells. For example, circulating immature NK cells found in the peripheral blood of WEHI-3B leukemic mice promoted immune suppression by decreasing the expression of $I\text{-}A^{d}$ on DCs via a TGF-β mediated process; decreased DC activity

also limited T cell activation (Ebata et al., 2006). Through these different mechanisms, NK cells can be both tumor-promoting and tumor-suppressing.

g. B cells

B cells are an adaptive immune lymphocyte involved in T cell activation and immune function maintenance. B cells are thought to play an indirect role in tumor immune surveillance. In mice challenged with B16 melanoma cells following B cell depletion, tumor proliferation and metastasis were enhanced, while $CD4^+$ and $CD8^+$ T cell proliferation was impaired (DiLillo et al. 2010). Thus, B cells confer a level of protection against cancer through induced T cell activity.

Other studies point to the immunosuppressive functions of B cells. B cells are also associated with the increased generation of Tregs. In a 4T1 TNBC mouse model, Bregs (a regulatory B cell population characterized by B2 markers, the activation of STAT3, and other markers) induce TGF-β dependent conversion of $CD4^+$ T cells into $FOXP3^+$ Tregs, and in the absence of Bregs, 4T1 is unable to metastasize to the lungs (Olkhanud et al., 2011). In tongue squamous cell carcinoma patients, $CD19^+IL\text{-}10^+$ Bregs transformed $CD4^+$ resting T cells into $CD4^+FOXP3^+$ T cells (Zhou et al., 2016). Both of these cases highlight the regulatory role Bregs may have in suppressing immune activity.

h. T cells

T cells have several functions including surveilling the body for non-self-antigens, helping B cells to produce antibodies, and eliminating harmful cells with toxic granules. They are also active in immune memory for future recognition of similar foreign bodies. T cells are self-tolerant, meaning they are unlikely to recognize self-antigens displayed on cancer cells as foreign antigens. However, cancer-specific neoantigens are tumor-specific and foreign antigens. Cancers that

display more neoantigens tend to have increased T cell infiltration, which is also modulated by other components of the microenvironment, such as MDSCs (Junttila and de Sauvage, 2013).

T cell populations include $CD4^+$ and $CD8^+$ cells and regulatory T cells or Tregs. In cancer heterogeneity studies, the distribution of these subtypes of T cells can influence outcomes. A comprehensive single-cell study performed on melanoma and the cellular tumor microenvironment found a diverse population of naïve, $CD4^+$, $CD8^+$, and Treg cells (Tirosh et al., 2016). Their analysis also revealed 28 genes that were consistently upregulated in high-exhaustion cells, including co-inhibitory receptors, T Cell Immunoreceptor With Ig And ITIM Domains (TIGIT), and co-stimulatory receptors 4-1BB and CD27 (Tirosh et al., 2016). Another patient with progressing metastatic ovarian cancer was found to have immune cell exclusion, while regressive and stable metastases had increased $CD8^+$ and $CD4^+$ T cells infiltration and exhibited oligoclonal expansion of specific T cell subsets (Jimenez-Sanchez et al., 2017). Jimenez-Sanchez and colleagues also detected $CD8^+$ T cell reactivity against predicted neoepitopes, after isolating cells from a blood sample taken three years after the tumors were resected. In a study evaluating ER^- basal and $HER2^+$ breast cancer using Boolean interaction Cox-regression models, the combination of high Th1 activation and low TGF-β activation defined a subtype with a good prognosis (Teschendorff et al., 2010). Given how much prognosis and tumor activity vary with distribution of T cell populations, the reduction of Tregs is a therapeutic approach that may improve patient outcomes. In mouse models, depleted Treg populations could restore NK function and promote T cell infiltration of tumors *in vivo* (Ghiringhelli et al., 2005). This suggests adoptive cell transfer (ACT) techniques that isolate T cells, amplify cytotoxic T cells, and reinfuse them back into patients could yield great benefits.

i. Non-Cellular Components of the Tumor Microenvironment

Beyond heterogeneity in the cellular components of the TME, some non-cellular traits that vary across tumors include the angiogenic, invasive, and metastatic potentials of cancers. These non-cellular alterations may be facilitated by the release of chemokines and growth factors from the cellular component of the TME (Watnick, 2012). For example, CAFs may reorganize the tumor and extracellular matrix by recruiting blood vessels to deliver nutrients to tumors. While increased blood flow can promote tumor growth, the increased vascularization may also be used to efficiently deliver therapeutic agents.

Spatial and Structural Heterogeneity

Cancers are often divided into subtypes based on their overall morphology and structure of origin. In breast cancer, the main structural categories include invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), which together account for 90% of breast cancer types (Li et al., 2005). Other types of breast cancers include mucinous, comedo, inflammatory, tubular, medullary, and papillary, and all these subtypes have variable age of diagnosis and different prognoses (Bertos and Park, 2011). These differences in prognosis may be due to the cells of origin, which have varied gene expressions. Another component of structural heterogeneity is the TME, which provides the architecture for particular tumors and their functions. A study evaluating the relationship between morphological subtypes of invasive micropapillary breast carcinoma (hollow-like, morula-like, solid, trabecular structures, and discrete groups of tumor cells) and cytogenetic heterogeneity found no link between chromosomal aberrations and morphological structures (Denisov et al., 2015). If chromosomal alterations have no influence on subtypes, then the role of the tumor microenvironment may be important in defining these subtypes, since this

study isolated pure tumor cells without admixture of non-tumor stromal cells (Denisov et al., 2015).

Some cancers have the same histology, but different structures of origin based on the cell population giving rise to the cancer. In lung cancer mouse model studies, selective gene KO models were made using adenovirus vector-mediated Cre recombinase systems. In SCLC, GEMM mice with triple KOs of TP53, RB1, and RBL2 established primary tumors in the proximal airways when the Ad-CGRP-Cre targeting construct was used, but established tumors in the distal airways when Ad-CMV-Cre targeting construct was used instead (Pozo et al., 2018). Another study found similar results in SCLC triple KO models: the Ad-CGRP-Cre construct resulted in proximal airway establishment in both the small and large bronchioles, while the Ad-CMV-Cre construct resulted in proximal and distal lesions forming in the terminal bronchioles and the bronchial-alveolar duct junction (Yang et al., 2018). Both of these cancers, irrespective of formation in the proximal or distal airways, are classified as SCLCs, and show structural heterogeneity is influenced by cell type of origin.

Spatial heterogeneity is a related concept and refers to how components of the tumor are distributed. Clonal populations often follow specific spatial patterns of distribution (Jiang et al., 2016, Yates et al., 2015). In metastatic melanomas, non-cycling cells were found to be clustered together and spatially distant from the clusters of cycling (dividing) cells (Tirosh et al., 2016). If cancers are grouped by clonal populations, tumor biopsies from one region may not reflect the diverse clonal makeup of the tumor, which is a limitation of current biopsy techniques.

Metastatic Heterogeneity

Metastatic heterogeneity addresses the difference in metastatic load on effector organs for cancers of the same histological origin. Observed metastases established at distal organs often

harbor distinct mutations and new phenotypes in comparison to the primary tumor site. Circulating tumor cells (CTCs) are often implicated in evaluating the metastatic potential of solid tumors as these cells are dislodged from their primary site and can establish a new mass elsewhere. CTCs that undergo the EMT, a process marked by increased expression of N-cadherin and vimentin, coupled with increased transcription factors like Zeb, Twist, and Snail, may be more likely to establish metastases (Jie et al., 2017). One of the earlier studies investigating CTCs evaluated the survival rates of (^{125}I)-5-iodo-2 deoxy-uridine–labeled B16 tumor cells and found under 0.1% of the initially injected cells survived when initial lung metastases were first established (Fidler, 1970). These findings raised the question of whether metastatic cells pre-existed in the initial population or if metastatic traits were acquired over time. In a follow-up study, parental B16 cells were divided into two parts with one half representing the parental population and the other half being used to establish 17 clones. The parental B16 cells and the 17 clones were injected intravenously in syngeneic C57BL/6 mice and the metastatic load was evaluated across groups. After 18 days, mice were sacrificed and evaluated for lung metastases, revealing a median number of 40 metastases formed from the parental cell line, but a range of 3 to >500 found across the metastatic clones (Fidler, 1978). Given the wide range of lung metastases, highly metastatic clones likely pre-existed in the parental population. These findings support the clonal evolution hypothesis, which will be discussed later.

Heterogeneity of metastatic tumor cell populations has been demonstrated in several key studies. In a pan-cancer whole-genome analysis of metastatic solid tumors, 2,520 pairs of normal tissue and metastases revealed the most commonly mutated genes to include TP53 (52% of samples), CDKN2A (21%), PIK3CA (16%), APC (15%), KRAS (15%), PTEN (13%) and TERT (12%), which collectively account for 26% of all the candidate driver mutations (Preistley et al.,

2019). The analysis also demonstrated that the mutational landscape and driver genes identified in metastatic tumors closely resemble the primary tumors; however, in 62% of patients, particular gene variants were identified to stratify patients towards additional drug therapies (Preistley et al., 2019). Understanding how metastatic cancers evolve over time is crucial to better prevent metastasis in early stages.

Macroenvironmental Heterogeneity

The tumor macroenvironment includes patient-specific factors such as age, body weight, and menopausal status (relevant for breast cancer in females) (Bertos and Park, 2011). While these factors are not tumor-specific, they may play a role with tumor-intrinsic factors. It should also be noted these factors are not necessarily independent from other forms of heterogeneity. Cancer is often considered to be a "disease of age" which can be attributed to the time required for cells to acquire the necessary driver mutations (Risques and Kennedy, 2018). Immune T cell exhaustion can also be acquired with age (Lee, K. et al., 2016) and accounts for the escape of cancers from immune surveillance (Wang et al., 2018). Increased body weight is correlated with increased estrogen levels in post-menopausal females, suggesting BMI may play a consequential role in breast cancer incidence rates (Bernstein and Ross, 1993). These factors are critical in defining personalized clinical care protocols.

II. Causes of Tumor Heterogeneity

Understanding the levels at which heterogeneity occurs can help determine what types of treatment strategies one should employ. It is equally important to understand how cancer heterogeneity may arise and be maintained. Currently, obtaining repeated biopsies from the same patient is not ethical, so most studies can only postulate how diverse populations of cancer cells

are maintained by constructing phylogenetic trees based on single biopsies (Davis et al., 2017). Two prominent proposals for the emergence and maintenance of tumor heterogeneity are clonal evolution hypothesis and the cancer stem cell hypothesis (Fig. 2). Both of these explain potential ways in which new populations of cells arise and form a heterogeneous tumor pool. Other evolution models address how successive new clones can be acquired and appear in a population. These include linear, branching, neutral, and punctuated evolution.

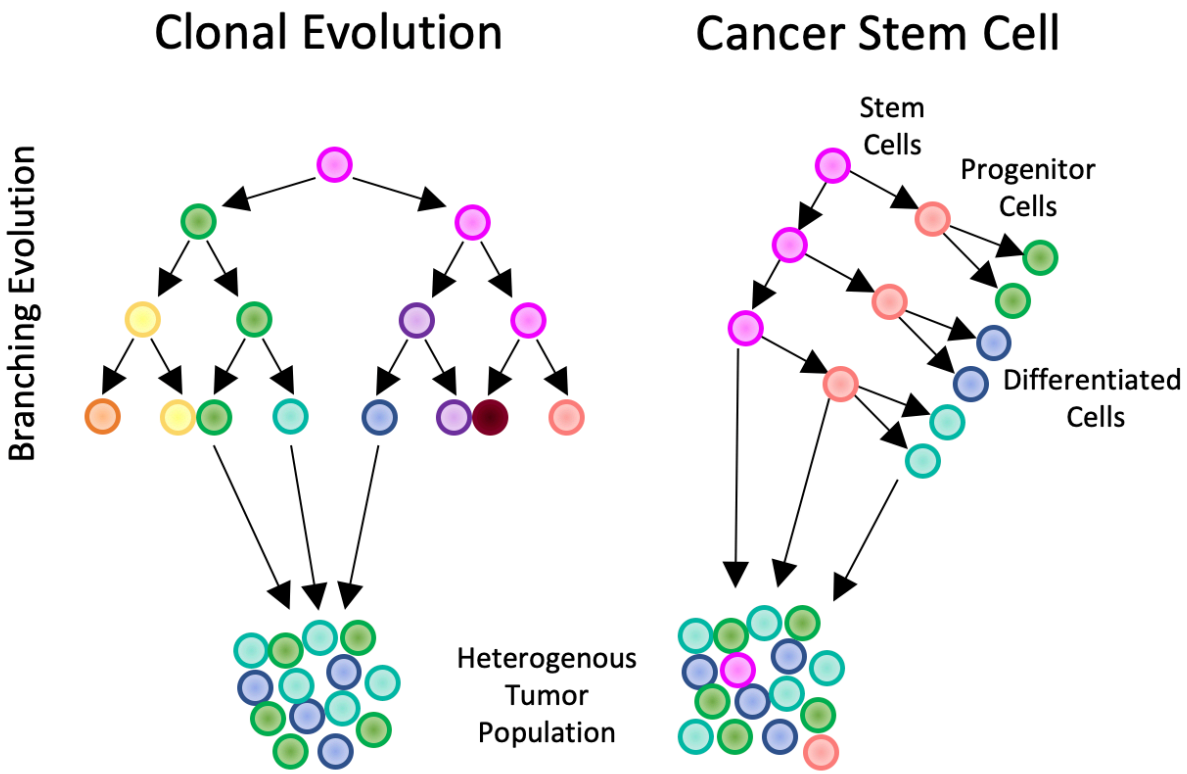

Figure 2. Clonal Evolution and Cancer Stem Cell Hypotheses. Under a branching evolution model of clonal evolution and cancer stem cell (CSC) hypotheses, a similar tumor population may be established. In clonal evolution, one stem cell can evolve into distinct lineages and the best adapted clones will contribute to be selected for the tumor population. Alternatively, in the CSC hypothesis, stem cells can self-renew and differentiate into distinct clones. The final tumor population contains the same admixture of clones plus the stem cells and progenitor cells.

Clonal Evolution Hypothesis

The clonal evolution model for tumor progression was first proposed by Peter Nowell in 1976. Due to underlying genetic instability and constant selection pressures from drug therapies and other limiting factors, such as poor nutrient availability to tumors, cancer cells are capable of giving rise to new mutants and clonal populations (Nowell, 1976). The clonal evolution model proposes there is one stem cell that initiates clonal expansion and, in turn, subsequent "hits" can be acquired overtime. The final heterogenous clones are the most aggressive, and the remaining clones die out (Hsiao et al., 2010). Early work supports this hypothesis, demonstrating that clonal diversity pre-existed in tumor populations (Fidler, 1978). Clonal evolution is often thought to follow a branching pattern that yields phenotypic differences in tumors. When evaluating histological samples of ER^+ tumors, there are connected clusters of ER^- cells that may have emerged under a clonal evolution model (Hsiao et al., 2010). The clonal evolution model suggests some subsets of a tumor have the potential for malignant growth from the start. It additionally suggests that clones can arise overtime from existing clones, presenting more challenges for proper treatment and management to overcome.

Cancer Stem Cell Hypothesis

In contrast with the clonal evolution hypothesis, the cancer stem cell (CSC) hypothesis suggests that only a certain subset of the tumor cell population is capable of contributing to cancer growth and progression. This subset, known as cancer stem cells, is capable of self-renewal and can maintain the tumor population (Michor and Polyak, 2010). There is also an inherent hierarchical organization of cells within a tumor, and the ability to expand and establish new clones is a property reserved for few multipotent cells. Although only CSCs can differentiate into more tumor cells, the CSC hypothesis believes all cells within the tumor can progress to a more

aggressive state (Hsiao et al., 2010). In lobular breast cancer, different lobules have distinct morphologies, suggesting that a separate stem cell forms each population (Hsiao et al., 2010). Beyond histological analyses, most CSC hypothesis validation has been performed in mice. One such study modeled human breast cancers in immunocompromised mice and found only 100 cells were tumorigenic (tumor-initiating) out of tens of thousands of cells that were non-tumorigenic or unable to establish tumors (Al-Hajj et al., 2003). The tumorigenic cells had a phenotype of $CD44^{+}CD24^{-}$ (Al-Hajj et al., 2003). Another mouse study performed with human colon cancer established that $CD133^{+}$ cells, which account for ~2.5% of the total tumor cell population, displayed tumorigenic properties, while $CD133^{-}$ cells were incapable of proliferating in immunodeficient mice (Ricci-Vitiani et al., 2007). Additionally, the characterization of marker expression profiles in distinguishing tumorigenic cells from non-tumorigenic cells implies that there is an epigenetic basis for separating CSCs from differentiated cancer cells (Shackleton et al., 2009). However, a limitation of these cancer stem cell validation studies is their reliance on immunodeficient mice. These mice lack the tumor microenvironment and immune surveillance present in humans. Even so, the CSC hypothesis has several important implications on drug screening and validation processes. Currently, one positive indicator of a promising drug therapy is the ability for it to reduce bulk tumor volume; however, if the CSC hypothesis is true and CSCs persist, these treatments may not prevent tumors from relapse. CSCs that are resistant to chemotherapy regimens are therefore potential targets for future and ongoing drug development approaches (Talukdar et al., 2016).

Other Hypothesized Models of Tumor Evolution

Other models of tumor evolution contribute to both the clonal evolution and cancer stem cell hypotheses. According to the linear evolution model, new mutations that give rise to clones

confer a selection advantage so strong that the new clonal population outcompetes the former clonal population and grows to fixation (Davis et al., 2017). In this model, only one clone can dominate, so the tumor population remains relatively homogenous but changes overtime with the acquisition of new mutations/traits. The linear evolution model was formulated around the idea of step-wise genetic mutations found in colorectal cancer (Fearon and Vogelstein, 1990). These early studies did not evaluate whole genomes, so there is a possibility their analyses missed other persistent clones.

Recent improvements in sequencing technologies reveal clonal patterns of inheritance that support the branching evolution model. This model suggests new clones emerge in parallel with other new clones, resulting in the presence of multiple clones at once. One explanation for why clones coexist comes from minimal differences in fitness (McGranahan and Swanton, 2015). Studies using next generation sequencing (Davis et al., 2017, Hardiman et al., 2016, Russnes et al., 2011), whole exome sequencing (Jiang et al., 2016, Mylonas et al., 2020, Tirosh et al., 2016,), and multi-region sequencing (Cao et al., 2015, Gerlinger et al., 2012, Yates et al., 2015) in conjunction with single-cell analysis present evidence in support of the branching evolution hypothesis. Branching evolution is one of the best supported models.

While branching evolution accounts for most situations, some cancers display alternate inheritance patterns. The neutral evolution model suggests no mutation or change confers any selective advantage, so all clones are represented proportionally. This contradicts the idea of driver mutations and the growth advantage attributed to them. Because some cancers do not have known drivers, this model might be at play (Shah et al., 2012). Another meta-analysis of different cancers found neutral evolution to be present in 323 out of 904 samples from 14 cancer types, which had early stage intratumoral heterogeneity (William et al., 2016). The study also developed a model

that inversely related intratumoral heterogeneity with allelic frequency, pointing to no selection advantage in clones (William et al., 2016).

In contrast to the past three models, the punctuated model of evolution proposes that heterogeneity in a population can arise all at once instead of sequentially over time. Supporting this, one study in prostate cancers found the majority of DNA translocation and deletion events were interdependent, suggesting they arose together (Baca et al., 2013). If the accumulation of mutations occurs in a coordinated moment, these findings support the punctuated evolution model. Another study found their phylogenetic analysis of a small subset of breast cancers with a majority of CNVs and chromosomal aberrations arose together in a short, punctuated burst (Gao et al., 2014). This model of punctuated evolution seems to explain linked genetic anomalies that relate to CNVs and may be useful in defining those clones across cancers. All four of these models about clonal evolution explain ways in which new clones may arise and be maintained in a population.

Genomic Instability

Another factor that can bring about changes in the genome and lead to new clones is genomic instability. Genomic instability is brought about by SNVs and CNVs in the genome, which can collectively result in greater cancer risk. These genetic alterations arise randomly, but the likelihood can increase through mutagens. DNA damage mutations can result from exposure to exogenous carcinogens like tobacco smoke, ionizing radiation, or UV radiation. Endogenous carcinogens such as free radicals can lead to replication fork collapse during meiosis and cause DNA mutations during replication (Roberts and Gordenin, 2014). Microsatellite instability (MSI) can arise from a decreased activity of MMR components. In a study evaluating primary and metastatic tumors from patients with sCRC, MSI was identified in 17% of these patients

(Jesinghaus et al., 2016). In squamous esophageal cancers, methylation of MLH1, a CpG island, was associated with MSI in most cases (Guo et al., 2006).

Beyond SNVs and CNVs, additional genetic alterations include those brought on by chromosomal abnormalities like translocation events or errors in segregation during cell division, resulting in aneuploidy. Chromosomal instability was determined to be a contributing factor in breast cancer, lung cancer, medulloblastoma, glioma, mesothelioma, and lymphoma (Carter et al., 2006). While translocations can result in CNVs contributing to gene amplification or suppression, not all translocation events account for a CNV. A notable example is the Philadelphia Chromosome that forms from the translocation resulting in ABL1 from chromosome 9 and BCR from chromosome 22 and forming a fused BCR-ABL gene on chromosome 22 (Salesse and Verfaillie, 2002). This mutant enzyme has unique catalytic functions on tyrosine kinase signaling and is found in chronic myeloid leukemia and acute lymphoblastic leukemia cases (Zheng et al., 2013). Other commonly implicated translocation events in lymphomas involve the insertion of genes into the heavy chain immunoglobulin gene locus on chromosome 14; some genes involved are the oncogene, MYC, found on chromosome 8 (Boxer and Dang, 2001), or the anti-apoptotic protein, BCL-2, found on chromosome 18 (Bakhshi et al., 2004). Chromosomal abnormalities are not often grouped with genetic heterogeneity, but they can be just as destabilizing to the genome and give rise to cancers. Understanding genomic instability can help explain the mechanisms that lead to heterogeneous tumor populations.

III. Methods for Evaluating Heterogeneity

New screening tools and bioinformatic approaches have revealed the heterogeneity across tumors. The newly identified targets can be used in diagnostic assays or as a new target for therapeutics. These approaches are important to assess heterogeneity with greater precision.

Tumor Heterogeneity Algorithms

Biomarker analyses are essential to characterizing tumors, identifying diagnostic and prognostic markers, and informing patient-specific therapeutic approaches. The mutant-allele tumor heterogeneity (MATH) scores can be used to quantify heterogeneity and act as a prognostic marker in various cancers. In one study of rectal cancer, MATH scores ranging from 7 to 41 were assigned to tumors, with higher scores effectively corresponding to poorer outcomes (Hardiman et al., 2016). MATH scores are also considered to be an effective prognostic marker in head and neck cancers, even with one tumor sample (Beck, 2015). The minimum event distance for intra-tumor copy-number comparisons, or MEDICC, is another algorithm developed for analyzing multiple samples in ovarian cancer. This algorithm has also been demonstrated to have clinical utility in measuring intratumoral heterogeneity from CNVs (Beck, 2015). The MATH and MEDICC algorithms were trained on different datasets, developed for specific cancers, and take different sequencing data inputs. Efforts to help standardize these and reduce bias can help increase their clinical relevance and use to further facilitate diagnosis and treatment.

Invasive Sampling Approaches

Thus far, tumor sampling has primarily consisted of biopsies obtained from tumor sites. The standard for tumor diagnosis is a single tumor biopsy, which is also used to determine the standard course of treatment for the cancer screened. However, new studies demonstrate the limitations single biopsies face, due to their poor representation of clones in a tumor mass. Further,

single biopsies provide limited information on how tumors evolve spatiotemporally in response to treatment. One team analyzed renal cell tumor samples from primary and metastatic tumor locations of four cancer patients, finding that 63-69% of mutations in single biopsies were not detectable across every tumor region in the same patient (Gerlinger et al., 2012). Another study of pancreatic cancer sampled 4-5 sites (lymph nodes) in patients, screened them for SNVs and CNVs, and found 90% intra-tumoral heterogeneity overall, suggesting the large majority of genetic anomalies harbored by the samples were private, with only 10% sample overlap (Cao et al., 2015). Additionally, evolution of cancers through space and time can result in dominance of different clones during the disease course and treatment (Swanton, 2012). Metastases may also vary in clonal makeup in comparison to the primary tumor. If clonal populations found across tumors are not representatively sampled, unidentified clones may persist. Sampling bias is a barrier to prognostics biomarker efforts.

It should be noted, not all studies point to such overt sampling bias from single biopsies. In an analysis of 2-3 spatially separated tumors per patient with rectal cancer, 67-97% of exonic somatic mutations were shared across all regions of an individual's tumor (Hardiman et al., 2016). Regardless, even if we evaluate the findings from Hardiman et al. conservatively, tumors that shared 67% of exonic mutations result in 1 in 3 unidentified clones. The underestimation of tumor heterogeneity may be ameliorated by increased sampling efforts.

Multiple biopsies may help increase clonal capture, but the invasive nature of tissue biopsies has potential disadvantages that must be weighed against the benefits of obtaining more tissue samples. There is limited evidence to support the cell seeding hypothesis, which suggests biopsies could dislodge tumor cells; however, there are preclinical studies that establish connections between wound healing and cancer. In murine mammary carcinoma models, increased

biopsy of mammary tumors increases the proportion of lung metastases, possibly through a IL-6 dependent inflammatory pathway, but blocking IL-6 activity can decrease the metastatic load (Hobson et al., 2013). In a clinical study of breast cancer patients, core needle biopsies included eosinophil recruitment and increased proliferation of tumor cells adjacent to the biopsy wound, suggesting biopsy-induced inflammation could impact tumor proliferation or metastasis (Szalayova et al., 2016). Components of the TME could be responsible for mediating the inflammatory response following biopsies at tumor sites, which could in turn lead to increased angiogenesis (Sundaram et al., 2018). More cohort studies could help to validate whether there are risks associated with biopsies in a specific cancer context.

Non-Invasive and Minimally Invasive Approaches

Noninvasive methods of tumor sampling are becoming increasingly relevant for diagnosis and for evaluating the effect of treatments overtime. Liquid biopsies may eventually replace tissue biopsies of solid tumor cancers. Methods for evaluating tumor burden and revealing the genetic makeup of a patient's cancer can be aided by noninvasive approaches. Circulating tumor DNA (ctDNA) may be used to assess tumor dynamics. While ctDNA quantities are lower in comparison to circulating cell-free DNA shed from noncancerous cells, increasingly sensitive detection approaches have mitigated this effect (Diehl et al., 2008). In a study monitoring 18 patients with colorectal cancers, plasma samples were obtained following surgery and analyzed through BEAMing, a highly-sensitive ctDNA detection tool (Diehl et al., 2008). The study found patients with complete resection had detectable ctDNA 2-10 days post-surgery, and patients with no recurrence were found to have undetectable levels of ctDNA from days 13-56 following surgery (Diehl et al., 2008). BEAMing was also able to identify mutations in APC, KRAS, TP53, and PIK3CA from ctDNA (Diehl et al., 2008). Finally, the study found ctDNA was a better predictor

of disease presence than carcinoembryonic antigen (CEA), and imaging tools were used to supplement the identification of small lesions (Diehl et al., 2008). Another study quantified ctDNA in NSCLC cases with cancer personalized profiling through deep sequencing (CAPP-seq) and found ctDNA corresponded to tumor burden, which was quantified through imaging (Newman et al., 2014). This study identified a median number of 4 somatic alterations that accounted for 96% of lung adenocarcinoma or squamous cell carcinoma (Newman et al., 2014). The study also detected ctDNA in 100% of patients with stage II–IV NSCLC and in 50% of patients with stage I, with 96% specificity for mutant allele fractions as low as ~0.18% (Newman et al., 2014). In an analysis of 30 women with metastatic breast cancer, 97% of patients had detectable ctDNA, 53% had ctDNA indicate treatment response (where increase ctDNA levels preceded the establishment of progressive disease), 78% expressed the prognostic markers, cancer antigen 15-3 (CA 15-3), and 87% had detectable CTCs (Dawson et al., 2013). Radiographic imaging was also used to assess the strength of ctDNA as a biomarker (Dawson et al., 2013). These studies all point to the use of ctDNA as a reliable and informative source of somatic aberrations. These findings indicate liquid biopsy approaches may be particularly helpful at diagnosing, and monitoring disease course overtime.

Imaging approaches can be useful for diagnosing and monitoring cancers at multiple timepoints, as imaging is a non-invasive method for confirming tumor burden and can supplement other tools which assess molecular markers of heterogeneity. Key examples of imaging modalities include X-ray, ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET). Within the MRI modality, a developing approach is the use of proton spectroscopic MRI (sMRI). In comparison to conventional contrast-enhanced MRI (ceMRI), metabolite data of biomarkers like choline (Cho) and *N*-acetylaspartate (NAA) in

glioblastomas was obtained to develop a more precise 3D tumor image (Cordova et al., 2016). Whereas ceMRI alone could not detect the contralateral tumors formed, the sensitivity of sMRIs allowed for more fine-tuned detection of metastatic brain lesions. Other solid tumor treatment strategies involve surgical resection of solid tumors. Obtaining a clean tumor mass with minimal residual tumor cells requires precise imaging techniques. If similar biomarkers can be found in other solid tumors, enhanced 3D imaging of tumor structures by sMRI could better predict prognostic outcomes in addition to providing information on the location of tumors.

IV. Therapeutic Approaches to Overcome Heterogeneity

Heterogeneous tumors contain several clones that drug therapy may target. These antineoplastics can place additional selection pressures on tumors and lead to decreased sensitivity and/or acquired resistance over time. Resistance mechanisms vary widely, making it difficult to develop target therapies. Understanding how to maintain sensitivity in tumors with high clonal diversity is imperative to improving cancer management and care.

Synthetic Lethality

One method of drug treatment is inducing synthetic lethality. This strategy uses drug inhibitors to target an already susceptible pathway (intrinsic susceptibility preexists due to genetic aberrations), in hopes that the redundancy will sensitize clones and lead to death. A well established example of synthetic lethality is the use of Poly (ADP-ribose) polymerase (PARP) inhibitors in patients with BRCA mutants. BRCA 1 and 2 are responsible for homologous recombination-mediated repair mechanisms. PARP proteins are involved in base-excision repair, another DNA damage repair pathway. In patients with BRCA deficiencies, treatment with PARP inhibitors can push the mutation load over some threshold and induce apoptosis of tumor cells

(Cerrato et al., 2016). Similarly, APOBEC-active cancers have a high mutation burden, and if patients are treated with DNA destabilizing agents, the tumors may accumulate enough DNA damage to induce cell death (Swanton et al., 2015). Another preclinical study suggests that inhibition of checkpoint kinase 1 (CHK1), a DNA -replication stress protein, could be a useful strategy in cancers with APOBEC3 induced mutagenesis (Kanu et al., 2016). More human trials evaluating the effect of DNA damaging agents can help target highly heterogeneous cancers.

Limitations of Target Therapies

Although the largely genotype-based approach has yielded initial success in targeted therapy and treatment, cancer cell populations tend to almost always acquire resistance over time. For example, in ER^+ metastatic breast cancer, when fulvestrant and tamoxifen are used together, resistant cells were found to harbor mutants in the binding domain of ESR1 (Jeselsohn et al., 2015). In the case of EGFR-dependent cancers, the third-generation tyrosine kinase inhibitors (TKIs) were developed to target EGFR T790M mutants, a common variant that arose in non-responders to first- and second- generation TKIs (Jänne et al., 2015). However, when third-generation TKIs were used upfront in EGFR T790M mutant tumors, patients who progressed developed a secondary EGFR C797S mutation (Niederst et al., 2015, Ramalingam et al., 2018). Further, admixture of clonal populations can be less responsive to targeted therapies aimed at a single clone, due to the presence of an intrinsically resistant clone (Song et al., 2014). Acquired resistance to drugs may also be at play for refractory tumors. Thus, while targeted therapies prolong progression free survival and overall survival, ultimately still result in recurrence. New approaches must factor in tumor heterogeneity to better control tumorigenesis. Finding more durable, long-term, or even curative solutions is crucial to overcome resistance to targeted drug therapies. Treatments designed

to target the immune microenvironment instead of the heterogeneous tumor population may help abrogate tumor resistance to targeted therapies.

Immune Checkpoint Inhibitors

One revolutionary advancement in cancer immunotherapy was the introduction of immune checkpoint inhibitors (ICIs). These include antibodies designed to block programmed cell death -1/-ligand 1 (PD-1/PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). PD-1 and CTLA-4 are exhaustion markers expressed on immune cells. When these IC receptors are activated by their complementary ligand, T cells are deactivated, resulting in cytotoxic T cell suppression and cancer evasion. Cancer cells express PD-L1, which is often a target of immunotherapy because it can suppress immune activity and promote immune evasion. T cell targeted modulatory drugs are approved for ~50 cancers (Robert, 2020), and immunomodulatory drugs account for 66% of oncology trials (Xin Yu et al., 2019). Further, one preclinical study found APOBEC3B expression increases resistance of melanoma cells to chemotherapy but increases sensitivity to ICIs. The susceptibility to ICIs can be attributed to increased neoantigen expression on the melanoma cells that T cells can target (Driscoll et al., 2020). For patients with metastatic melanoma treated with pembrolizumab, an anti-PD-1 monoclonal Ab (mAb), durable complete response was noted in ~15% of the cohort after discontinuing treatment for nearly ten years (Robert et al., 2018). ICIs therefore have a curative effect on various cancer types; however, despite these accomplishments, ICIs are only effective in a fraction of patients. This suggests that ICI non-responders may have poor or no pre-existing T cell immunity for the ICI to act on (Pardoll, 2012). While ICIs may disable the “brakes” that suppress immune activity, T cells must be stimulated with some other “fuel.” This “fuel” could be provided through vaccination.

Whole Cell and Membrane-Based Vaccines

Vaccines prime the immune system by introducing antigens to naïve T cells, triggering the activation and rapid differentiation of effector T cells (Chen and Mellman, 2013). These antigens can be presented to T cells via MHC Class I or II complexes, resulting in priming and activation of effector T cells. These effector T cells must then infiltrate tumors, selectively bind to cancer cells with T cell receptors (TCRs) and kill the target cancer cell (Chen and Mellman, 2013). This model of immune-activated cancer clearance is called the "cancer-immunity cycle," for which several steps must occur to generate a successful vaccine response.

It should also be noted that vaccine approaches to cancer treatment were common before ICI therapy in the clinic. Prior to ICIs, solid tumor immunotherapy relied on peptide immunizations, viral vaccines, and allogeneic whole cells that secreted specific immunocytokines (Rosenberg et al., 2004, Srivatsan et al., 2013). One barrier to the development of immunogenic cancer vaccines is self-tolerance. Since cancer cells originate from healthy cells, they share the antigen repertoire found on regular cells, and vaccines aimed at self-antigen are not as immunogenic as vaccines aimed at foreign infectious agents, like bacteria and viruses (Selvaraj et al., 2008). Additionally, these vaccines often have limited success in humans for a number of reasons. Firstly, whole cell vaccines developed with radiation often express phosphatidylserine, a immunosuppressive marker typically found on the inner leaflet of a cell membrane, leading to immunosuppressive activity (Srivatsan et al., 2013). These effects can be mitigated through the incorporation of immunostimulatory molecules. Further, even if vaccines were able to generate cytotoxic T cells, they may not be able to infiltrate solid tumors. This lack of infiltration may limit successful outcomes in vaccine trials for liquid tumors, such as lymphoid cancer (Rosenberg et al., 2004). Lastly, allogeneic vaccines offer a platform for developing mass produced agents that can

treat multiple people. However, even if broadly available, patient-to-patient heterogeneity and intratumoral heterogeneity would present challenges to allogeneic vaccine approaches.

Autologous vaccines, derived from self-tumor, can improve outcomes. A personalized vaccine approach develops vaccines that reflect the heterogeneity of an individual's tumor. Since these vaccines are personalized, developing these vaccines may require more time than a patient has, but may significantly improve clinical outcomes. One such personalized cancer vaccine model is the tumor-membrane vesicle (TMV) vaccine. TMVs are plasma membrane vesicles derived from tumor tissue that is homogenized to make vesicles ranging from 300-600 nm (Patel et al., 2015). TMVs carry many tumor-associated antigens. TMV vaccines are prepared by incorporating immunostimulatory molecules (ISMs), such as B7-1 and IL-12, by protein transfer (Patel et al., 2015). The TMV vaccine model has been evaluated in syngeneic mouse models of breast cancer and has been shown to induce strong anti-HER2 responses (Patel et al., 2015). TMV vaccines have also conferred protection in mouse models of TNBC in combination with anti-CTLA-4 mAb (Pack et al., 2020) and head and neck cancer with anti-PD-1 mAb (Bommireddy et al., 2020). TMV vaccines are also useful because the tumor cell membrane components are still present, eliminating the need for live cell vaccines (Cimino et al., 2004). This vesicle-based model provides more control over the response elicited by the vaccine.

Dendritic Cell Vaccines

When considering the cancer-immunity cycle, DC vaccines are known to bypass the protein-loading step in vivo, by pulsing DCs with tumor antigens ex vivo. DCs are also the most potent antigen-presenting cell, and tumor antigens loaded onto DCs can be effective for priming T cells. One prominent antigen-presenting cell-based vaccine is Prostate GVAX, which consists of irradiated allogeneic prostate tumors with GM-CSF secretion to treat hormone-refractory prostate

cancer (HRPC). The GVAX trial results showed the vaccinated group had a 26-month overall survival improvement over the control group, which was treated solely with taxane chemotherapy (Small et al., 2007). Given that whole tumor cell vaccines can secrete pro-tumor cytokines like IL-10 and TGF-β, DC vaccination approaches with cell lysates are of interest (Chiang et al., 2015). For example, a preclinical study with mice obtained cell lysates from mammary carcinoma and compared them to DC vaccines that were developed using whole fibrosarcomas; the study found both methods induced $CD8^+$ and $CD4^+$ activation, but the cell lysate vaccine protected the mice from lung metastases more than the whole cell vaccine (Fields et al., 1998). Furthermore, protein and RNA-based DC loading are favorable because neither antigen source has HLA restriction. With DC loading, the RNA can also be converted to cDNA ex vivo and loaded onto DCs. $CD8^+$ T cells recognize the antigenic peptides presented on DCs and induce an immune response (Mitchelle and Nair, 2000), suggesting DNA/RNA-loaded DCs may be more effective. An autologous monocyte-derived DC study electroporated mRNA of glioblastoma cancer stem cells. This study demonstrated 2.9-fold increase in progression-free survival in comparison to the control group (Vik-Mo et al., 2013). In addition to its use in studies, an RNA loaded vaccine approach is also beneficial as it expands the number of antigens that may be targeted. These antigens could be associated with tumor stromal components rather than the tumor cells themselves (Chiang et al., 2015). DC based vaccines development approaches are therefore more widely applicable to cancers due to the number of targets they can display without the need for HLA-matching.

Adoptive Cell Transfer

While loading DCs ex-vivo can enhance antigen uptake and increase the chance of T cell activation, ACT aims to expand select T cell clones ex vivo. In ACT, cytotoxic T cells are modified to express engineered immune receptors with antibodies and T cell co-receptors called chimeric

antigen receptors (CARs). These modified T cells are then reinfused into patients as a treatment. CAR T cell therapy has been used to treat acute B lymphoblastic leukemia (ALL) by targeting CD19 antigen expressed on ALL tumor cells (Lee et al., 2015). However, similar target proteins or genetic anomalies have yet to be discovered and utilized for other cancer types.

TCRs are naturally occurring "CAR-like" receptors that bind to MHC molecules presenting peptides derived from antigens. One research group looked into developing T cell receptors (TCRs) against tumor-associated antigens in melanoma. In the study, melanoma tumors with HLA-A2 receptors bound to either CD20 or myeloperoxidase were co-cultured with HLA-A2-negative donor T cells. Healthy donor T cells from HLA-A2-negative patients were used, since HLA-A2 would be considered a foreign peptide, prompting TCR generation against HLA-A2. Cytotoxic T cells were generated against 37 out of 50 peptides predicted to bind HLA-A2, with 36 new epitopes previously undescribed (Kumari et al., 2014). The study demonstrates more self-antigens can be targeted for new ACT approaches. While this study was performed in a liquid tumor cancer, expanded T cell clones against epitopes like HER-2 or prostate-specific membrane antigen (PSMA) may be targeted for ACT treatment (Kailayangiri et al., 2020).

In addition to tumor-associated antigens, neoantigens are also promising treatment options, as these markers are unique to cancer cells and limit the off-target impact on normal cells. However, neoantigens are expressed in low levels across cells in a tumor population, which pose a challenge to their use in treatments (Nejo et al., 2019). Another study looked into neoantigen-driven T cell immunotherapy (Stronen et al., 2016). The study developed a predictive model for finding the most immunogenic neoantigens. After obtaining whole exome and RNA-seq data and identifying SNVs, the study employed an MHC I binding algorithm to narrow down the most promising neoantigens. These neoantigens were then used in binding assays along with

allogeneic HLA-matched donor T cells and found neoantigens with the most immune-promoting potential displayed the strongest binding interactions between the neoantigen peptide and MHC I (Stronen et al., 2016). Since neoantigens are unique to each patient, developing T cell populations against commonly acquired cancer mutations, like EGFR variant III (EGFRvIII, in-frame deletion of exons 2–7), would benefit the larger population (Goff et al., 2019). Another common mutation includes the KRAS G12X mutants (where X represents amino acids C, V, D, A, and S) found in lung adenocarcinomas, which may elicit T cell reactivity (Calvayrac et al., 2017). The findings from Stronen et al. indicate peptide-MHC complex stability could be an additional immunogenicity criterion (Yadav and Delmarre, 2016). This knowledge could shorten the development time of personalized vaccine therapies.

Oncolytic Viruses

Viruses are efficient and highly specific infectious agents. Their machinery can be harnessed to infect cancer cells. Oncolytic viruses are engineered to selectively target cancerous cells and lyse them. The lysing of cancer cells serves two purposes: firstly, to destroy the cancer cells, and secondly, to induce anti-tumor immunity (Melcher et al., 2011). Oncolytic viruses can also facilitate the delivery of additional immune bolstering components, like cytokines and antibodies (Goradel et al., 2019). The first FDA approved agent was talimogene laherparepvec (T-VEC), which is a herpes-based melanoma-targeting virus that lacks ribonucleotide reductase and expresses GM-CSF (Conry et al., 2018).

A major limitation to oncolytic viral therapy is resistance. In a two-year immuno-oncology summary report, oncolytic viral therapies had the smallest growth in new treatments, with only 35 new approved oncolytic agents (Xin Yu et al., 2019). In B16 melanomas, the introduction of oncolytic vesicular stomatitis virus led to the upregulation of APOBEC3 in an IFN-β-dependent

manner (Huff et al., 2018). To overcome these resistance mechanisms, researchers can use combination treatment with other immunotherapeutics. One study looked at combining a viral agent with ICO15K-FBiTE, a fibroblast-targeting bispecific T cell engager, to enhance viral spread and T cell mediated cytotoxicity against CAFs (de Sostoa et al., 2019). Choosing the right viral vector for the specified target is also critical to success. For example, naked viruses are more successful than enveloped viruses (Zheng et al., 2019). Overall, oncolytic viruses offer a precise tumor-targeting system, but there are limitations to their potential. As such, combination therapy with an oncolytic virus may be more efficacious. While oncolytic viruses directly target cancer cells, they can indirectly target the immune system and promote an anti-tumor response. The anti-tumor immune response can be useful in clearing cancer cells which were not directly targeted by the therapy, which may occur due to tumor heterogeneity.

Conclusion

A strong understanding of tumor heterogeneity informs the drug development pipeline. With so many treatment options and different classes of drugs, it is important to develop more robust informatics frameworks to better grasp the nuances of heterogeneity. These could be used to identify combinations of clonal characteristics that correspond to more favorable prognostic outcomes. Similarly, these networks could be used to determine which combinations of drugs yield better outcomes. For example, cases with high genetic aberrations from DNA MMR have improved outcomes when more DNA damage is induced (Lee, V. et al., 2016). Understanding where this threshold for damage falls is a concept that needs to be defined more concretely.

Studies evaluating cancer heterogeneity must also take the effects of the microenvironment into account. There are limited available studies on the relationship between tumor heterogeneity

and tumor infiltrating lymphocytes (TILs). TILs may be a useful prognostic marker for treatment outcomes and these cells may be used in adoptive cell transfer for treatment.

When considering how clonal diversity can evolve, identifying specific patterns of mutation inheritance could help inform treatment approaches. For example, under the punctuated evolution model where several interrelated changes occur simultaneously, the formation of mutation clusters may better predict which types of genetic variants are harbored in the bulk tumor, especially when single biopsies often fail to capture clonal diversity.

Improving clinical guidelines for the evaluation of efficacious cancer immunotherapy is a needed change, since current guidelines are not well adapted to the rapid surge of these treatments. For example, many phase I trials of immune-oncology agents include expansion cohorts to treat more people with the agent even if it is not formally approved for the treatment of those disease-cohorts (Siu et al., 2017). While it is great more patients are treated with promising agents prior to trial completion, the lack of rigorous scientific validation and close monitoring of patients in these cohorts may cause more harm to those patients (Siu et al., 2017). Additionally, trials have various markers of success such as progression-free survival, overall survival, overall response rate, and these differences make it difficult to generalize conclusions from trial to trial. Given how critical immunotherapies are to cancer treatment management, developing clear parameters to objectively define treatment outcomes would decisively advance the ever-growing cancer treatment body of knowledge.

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