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Tumor Heterogeneity and Therapeutic Challenges: Exploring Approaches and Future Directions

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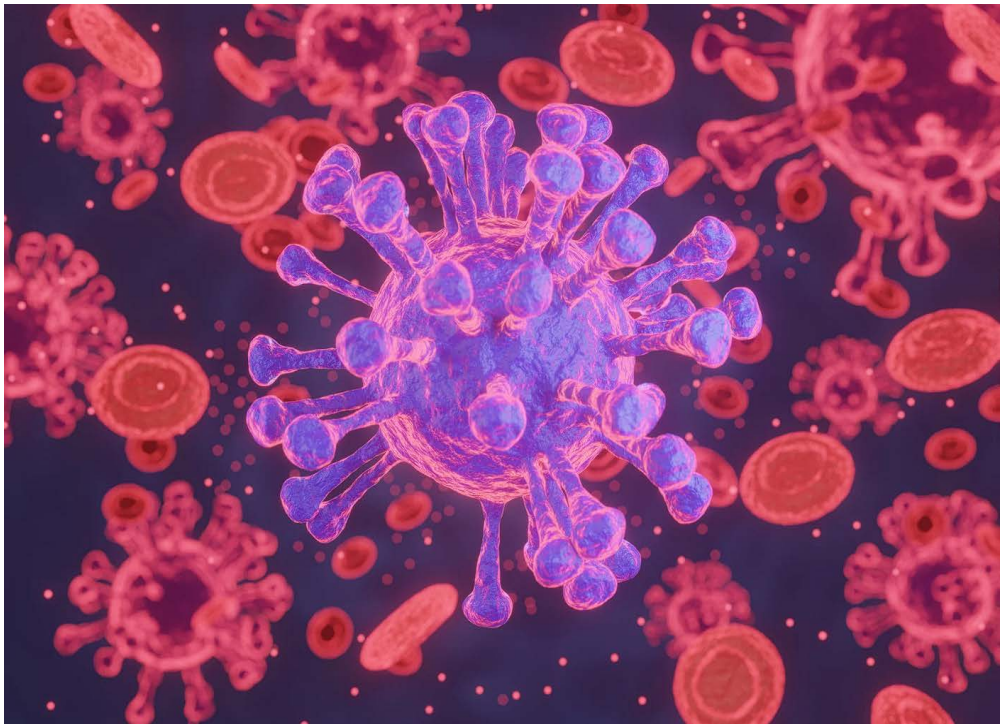

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Abstract

Tumor heterogeneity refers to the phenomenon when tumors possess a medley of cell types, and it is one of the central challenges facing cancer treatment as it is associated with drug resistance and worse prognosis. Each cell type responds to treatment in a unique way, thus tumors with high levels of heterogeneity are not often fully treated by typical cancer therapies. There are a variety of novel treatments being developed that aim to eliminate the obstacle that heterogeneity poses by utilizing more personalized approaches. This review assesses immunotherapy, combination therapy, dual- or multi-ex vivo armed T cells, and a nano-Cas9 ribonucleoprotein system as treatment strategies. Although further research is required to ensure their clinical safety, these treatments show their potential to overcome the challenges posed by tumor heterogeneity in cancer development. This paper also discusses circulating tumor cells as a way to test therapeutic drugs and determine treatment progress.

1. Introduction

Cancer, a disease in which cells grow uncontrollably, was one of the three leading causes of death in 2022, with 607,790 deaths in the U.S.[1] In 2023, an estimated 609,820 out of roughly 2 million people in the U.S. were predicted to die of cancer.[2] However, treating cancer is rather complicated. Cancer is not merely one disease but rather a group of diseases that consist of various classifications, with each cancer generally named after the body part where the abnormal cell growth begins. The various types of cancers share certain hallmarks, such as invasion and metastasis, tumoral angiogenesis, and the evasion of apoptosis.

The most common cancers in the U.S. are breast, prostate, and lung cancer, but it can also occur in skin cells or bone tissues.[3] The problem is that no two cancers are exactly alike, even if they are classified as the same type or occur in the same body tissue. Each individual's cancer contains unique combinations of genetic changes, which can affect a patient's response to specific treatments.[4] Researchers from the Wellcome Trust Sanger Institute analyzed 4,938,362 mutations from 7,042 cancers, investigated the genomic dynamics of tumors undergoing exogenous and endogenous mutational processes, and extracted more than 20 distinct mutational signatures.[5] These statistics highlight the vast possibilities of genetic alterations a cancer cell could undergo, leading to more variation between and within tumors. Factors such as family history and lifestyle habits, especially smoking, also contribute to the uniqueness of each individual's tumor. Hence, this individuality of cancer, also known as tumor heterogeneity, complicates its treatment.

Tumor heterogeneity refers to the disparities between tumors of the same type in different patients, between cancer cells within a single tumor, or between primary and secondary tumors. Intratumor heterogeneity (ITH), which refers to the presence of a diverse cell population within a single tumor, poses the greatest challenge to cancer treatment today. It can be further distinguished by genetic and phenotypic properties, which vary and affect the behaviors of different tumor cell populations. One system of tumor cell analysis is the Clustering, Classification, and Sorting Tree (CCAST), which aims to target specific characteristics that can vary widely

within malignant cells. It analyzes the genetic and phenotypic variations within tumor cells by distinguishing homogeneous subpopulations within a mixed group of single cells. For example, CCAST was applied to a breast cancer cell line and identified at least five distinct cell types, which helped elucidate which tumor cell subpopulations warranted further investigation.[6] Thus, utilizing such diagnostic guidelines to identify variations in genetic and phenotypic properties is beneficial as a prognostic indicator to better predict tumor response and guide therapeutic precision strategies. Associated with poor prognosis, outcome, and overall survival, ITH is thought to be a significant factor in causing therapeutic resistance and treatment failure.[7] Tailoring effective cancer treatments is also challenging due to the unique response patterns exhibited by individual patients. Thus, understanding tumor heterogeneity is crucial in treating cancer and overcoming therapeutic resistance.

Initially, the primary source of tumor heterogeneity was thought to be genetic or epigenetic alterations as scientists were beginning to understand the impact of tumor heterogeneity.[8] While this understanding holds some truth, more studies in recent years have observed tumor heterogeneity of varying types, such as metabolic, cellular, spatial, and more. However, despite the efforts of current common cancer treatments to address tumor heterogeneity, there is still a significant risk of cancer cells resisting treatments and therapies through mutations, selective pressure, and other adaptation processes, setting back the development of more effective cancer treatments.

The latest endeavors have focused on developing novel strategies to overcome the challenges posed by tumor heterogeneity and combat treatment resistance and disease progression. Some of these treatments target unique features of the tumor, allowing for a more precise and personalized approach. We reviewed recent scientific literature for an overview of tumor heterogeneity, along with current and potential cancer treatments that surmount its challenges. In addition, we examined recent and ongoing clinical trials to analyze the purpose and design of the studies and gain more information on the significance of their results. Through this review, we hope to provide insight into the causes and negative effects of

tumor heterogeneity on individual cancer treatments, along with potential strategies that could overcome such negative implications.

2. Tumor Heterogeneity

2.1 Overview of Tumor Heterogeneity

Tumor heterogeneity has countless causes that complicates its prevention methods and treatment, with two of its leading causes being genetic and environmental factors. Table 1 expands on the causes of tumor heterogeneity, including genetic mutations, clonal evolution, microenvironmental factors, and phenotypic plasticity.

Factors	Genomic Instability	Clonal Evolution	Microenvironmental Factors	Phenotypic Plasticity
Definition	The higher tendency of cells to obtain genetic alterations[9]	The process by which different subclones within a tumor change over time[11]	Factors (e.g. oxygen, nutrient availability, immune cell infiltration) and interactions with surrounding stromal cells in the tumor microenvironment	The ability of one genotype to cause different phenotypes to arise in response to different environments[15]
Causes	Anything that causes more genetic mutations (e.g. limitless replicative potential[10], DNA repair defects[9])	- Typical evolutionary processes[12] - Influenced by many factors, such as the tumor microenviron-ment, metabolism, growth factors, and mutation	- Collection of tumor cells that combine to create the tumor microenvironment[13] - Consists of the extracellular matrix, immune cells, and stromal cells[13]	- Epithelial-to-mesenchymal transition (EMT) and certain transcription factors inducing EMT (e.g. Zeb1, Twist)[15] - Genetic mutations and epigenetic

		rate[12]		modifications
Effects	- Results in genetically distinct subpopulations of different cells → Genetic heterogeneity	- Leads to the expansion of specific populations of tumor cells[11] → Cellular heterogeneity	- Promotes angiogenesis and an environment for cancer cells to grow[14] - Prevents immune cells from infiltrating the tumor[14] - Influences surrounding cells and cancerous cells[14]	- Protects metastasized cancer cells from ferroptosis, a type of cell death[16] - Give rise to subpopulations of tumor cells with distinct functional properties[16] → Cellular heterogeneity

Table 1. Factors contributing to tumor heterogeneity. This table summarizes the definition, causes, and effects of the different factors that contribute to tumor heterogeneity–from left to right: genomic instability, clonal evolution, microenvironmental factors, and phenotypic plasticity.

There are four types of tumor heterogeneity: intratumor, intermetastatic, intrametastatic, and interpatient. All four types cause unique complications in the clinical process. This review focuses on intratumoral heterogeneity, which refers to phenotypic or genetic variation in cells within a single tumor. The other three types of tumor heterogeneity offer additional context to the complexities of tumor heterogeneity. For instance, intermetastatic heterogeneity is the variety between two metastatic tumors within the same patient; metastatic tumors are two or more tumors that differ from one another. Intrametastatic heterogeneity is ITH within a metastasis – there is variation within that single lesion. Lastly, interpatient heterogeneity is the difference in tumors between different patients possessing the same type of cancer. Interpatient heterogeneity is the main reason for the need of personalized treatments, regardless of the level of variation observed within a single tumor.[17]

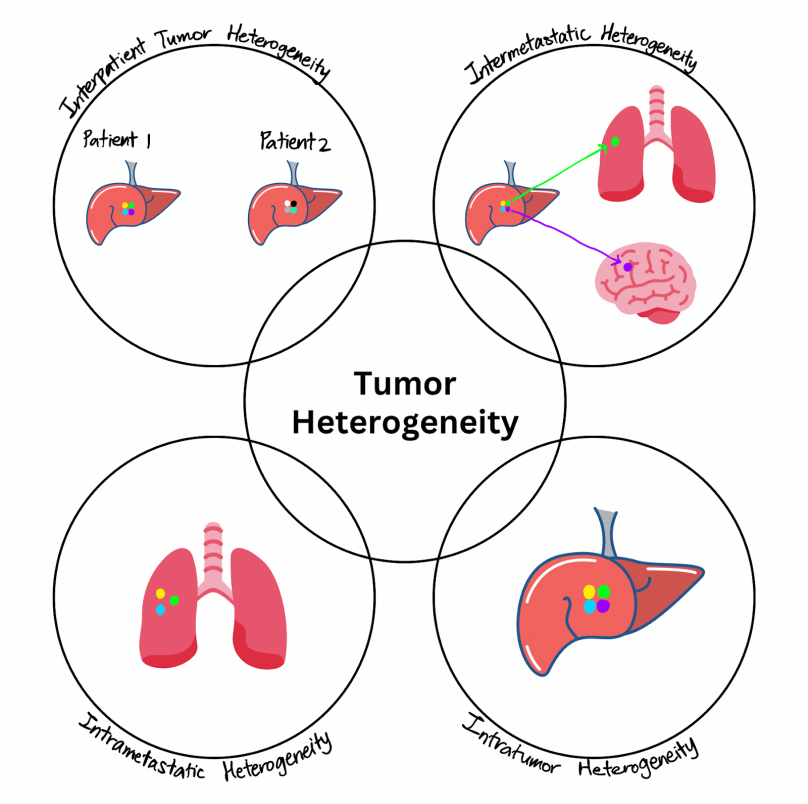

Figure 1. Types of tumor heterogeneity. The different-colored dots represent different subpopulations of cells in a tumor – in other words, tumor heterogeneity. The top left circle represents interpatient tumor heterogeneity between two patients with tumors in their liver, where despite having the same cancer, their tumors have unique subpopulations of cancer cells. The top right circle shows intermetastatic heterogeneity in a single patient. The diagram illustrates how different subpopulations of cancer cells from the liver metastasize to the lung and the brain, resulting in the presence of tumor heterogeneity between the different metastatic lesions. The bottom left circle displays intrametastatic heterogeneity, where different subpopulations of cancer cells are present within a lung metastasis. Lastly, the bottom right circle shows intratumor heterogeneity in the liver of a single patient, where the primary tumor contains different subpopulations of cancer cells.

2.2 Intratumoral Heterogeneity Across Different Types of Cancers

Intratumoral heterogeneity can reflect genetic alterations in the tumor, which can affect disease progression and treatment response. For example, mutations in genes like BRCA1, BRCA2, TP53, and HER2 in breast cancer were identified as risk factors in cancer development and can impact treatment outcomes.[18] Additionally, in lung cancer, mutations in epidermal growth factor receptor (EGFR) and KRAS, along with ALK and ROS1

rearrangements, are significant determinants of tumor behavior and therapeutic choices.[19,20] Identifying these genetic variations allows physicians to accommodate treatment plans to tumor-particular genetic structures, resulting in more effective treatments and improved patient outcomes. One method that targets these genetic mutations is CRISPR-Cas9-mediated genome editing, which can be utilized to target specific alleles, such as the TP53 gene in KHOS and KHOSR2 cell lines of osteosarcoma. This technology can hinder tumor proliferation and migration, as well as modify drug sensitivity in cancer treatment.[21]

Cellular heterogeneity in ITH refers to the numerous subpopulations of cells inside a tumor, each with precise traits and sets of behaviors. For instance, cell types in breast cancer include luminal cells, basal-like cells, and HER2-positive cells, all of which impact general tumor behavior due to different signaling pathways and levels of receptor expression. [22,23] Understanding cellular heterogeneity helps researchers and clinicians identify potential targets for therapy since different cell populations may respond differently to various treatments. Targeting particular cellular subpopulations would disrupt a tumor's growth and development more effectively. Table 2 provides a comprehensive overview of the genetic and cellular heterogeneity observed in key cancer types.

Cancer Type	**Genetic Heterogeneity**	**Cellular Heterogeneity**
Breast Cancer	BRCA1, BRCA2, TP53, HER2/neu mutations[18]	Luminal cells, basal-like cells, HER2-positive cells[22,23]
Lung Cancer	EGFR mutations, KRAS mutations, ALK rearrangements, ROS1 rearrangements[19,20]	Adenocarcinoma cells, squamous cell carcinoma cells[24]
Colorectal Cancer	APC, KRAS, TP53, BRAF mutations[25]	Stem-like cells, differentiated cells, tumor-infiltrating lymphocytes[26]

Prostate Cancer	PTEN, TP53, AR alterations[27]	Adenocarcinoma cells, neuroendocrine cells, cancer stem cells[28,29]
Bone Cancer (Osteosarcoma)	TP53, RB1, p16INK4a variations[30]	Osteoblastic cells, chondroblastic cells, fibroblastic cells[31]

Table 2. Genetic and cellular heterogeneity of different types of cancers. This table illustrates genetic and cellular heterogeneity of different types of cancers. Column 1 identifies cancer types, column 2 indicates genetic heterogeneity, and the last column shows cellular heterogeneity.

The presence of ITH across these different types of cancers emphasizes the significance of personalized treatment approaches. Understanding a tumor's unique genetic and cellular landscapes can guide the selection of appropriate therapies that target specific cell populations or overcome resistance mechanisms. Integrating technologies like single-cell analysis and spatial profiling techniques allows for a comprehensive assessment of ITH and aids in the development of tailored treatment strategies.[37]

2.3 Determining the Degree of Tumor Heterogeneity

A tumor's degree of heterogeneity is correlated with tumor prognosis, genomic instability, tumor advancement, and immunosuppression. Higher levels of heterogeneity (i.e. a tumor with a greater cell diversity) suggests that the patient is more likely to experience worse treatment outcomes and drug resistance. Knowing the level of heterogeneity a tumor possesses permits researchers to begin planning specialized treatments since more diversity in the cell population demands a more personalized treatment. This knowledge also permits studies to be done on groups with high and low levels of heterogeneity in order to determine the impact of ITH on a specific treatment.[36]

Research has determined that three methods are the most accurate and effective regarding how closely correlated they are with heterogeneity

outcomes: DEPTH[38] , DEPTH2[39] , and tITH[40], which all have comparable performances. These newer algorithms differ from those used in previous studies in that they implement RNA sequencing as opposed to DNA sequencing. Experiments using DEPTH, which stands for Deviating Gene Expression Profiling Tumor Heterogeneity, were conducted for over 25 cancer types alongside 10,000 samples of TCGA pan-cancer. DEPTH showed stronger correlations between tumor prognosis and anti-tumor heterogeneity than DNA-based algorithms such as ABSOLUTE, EXPANDS, MATH, and phyloWGS.[38] DEPTH2 showed similar correlations to the original DEPTH program, but it had the advantage of being applicable to more gene expression profiles as it does not reference normal controls like other mRNA or DNA-based algorithms.[39] DEPTH2 was used in a recent 2022 study to separate groups into high and low intratumor heterogeneity groups. These groups were then compared in a study to determine a correlation between heterogeneity and chemotherapy response in patients with colon adenocarcinoma.[41] Finally, transcriptome-based ITH (tITH) involves defining a network and determining the distance between typical genetic sequences and cancer sequences. Pathway-tITH is defined using the genes from one specified pathway. One study demonstrated that in 255 out of 291 pathways, genomic ITH was strongly correlated with pathway-tITH, supporting earlier findings that overall genetic diversity impacts variation in certain pathways.[40] Table 3 below displays information on the DNA-based algorithms used in earlier studies, as well as the more recent RNA-based algorithms primarily discussed in the paper.

Algorithm Name	**Description**	**Features**	**Year Created**
ABSOLUTE[42]	Profiles DNA from heterogeneous cell populations to determine cellular copy number and identify variant alleles	- Identifies alterations in cancer cells - Evaluates tumor ploidy estimates	2012

MATH[43]	Measures intratumor genetic heterogeneity based on mutant-allele fraction	- Correlates ITH with mutations in TP53 and HPV status	2013
EXPANDS[44]	Characterizes coexisting tumor subpopulations using copy number and allele frequencies	- Estimates tumor purity and predicts clonal subpopulations - Quantifies genetic ITH	2014
PhyloWGS[45]	Combines somatic mutation and copy number information for subclonal reconstruction	- Provides complete subclonal reconstruction	2015
tITH[40]	Models gene relationships and measures network disruptions to assess ITH	- Shows positive correlation with tumor progression and worse survival	2016
DEPTH[38]	Calculates ITH based on gene expression profiles from RNA sequencing data	- Is associated with genomic instability, worse survival, and decreased antitumor immunity	2020
DEPTH2[39]	Calculates ITH based on disruptions of gene expression profiles without reference controls	- Is associated with worse survival and more aggressive cancer subtypes	2022

Table 3. Algorithms used to assess tumor heterogeneity. This table lists the algorithms that are used to assess tumor heterogeneity in order of the year it was created. It also explains how each algorithm works and what it assesses.

Single-cell RNA sequencing (scRNA-seq) has also shown promise in helping develop personalized treatment plans. ScRNA-seq works by isolating the tumor cells and running mRNA reverse transcription and

cDNA amplification before sequencing. As recent as 2020, single-cell RNA sequencing efficiently analyzed thousands of cells at once, making it more useful for treatment than in previous years. A study found that scRNA-seq could adequately determine clusters of cells associated with poor clinical outcomes and find targets for immunotherapy treatments in an analysis of triple-negative breast cancer (TNBC). Knowing the different types of cells in a tumor will become essential in the personalized treatment for high-degree heterogeneous tumors, which will need a combination of therapies in order to be effectively treated. There are, however, some limitations to this technology, including cell integrity and viability, its relatively high cost, and its integration with other genomic and protein information. Further research to advance scientific and technological developments, such as gentle extraction and data analysis methods, is expected to overcome these challenges.[46]

2.4 Complications of Tumor Heterogeneity

Different cell types respond to different treatments, which is the reason behind the utilization of combination therapies for tumors with high degrees of heterogeneity. Using a single treatment can lead to a relapse of cells that are more resistant to typical therapies. The first round of treatment might effectively eliminate one type of cell, making the tumor appear smaller. However, over time, the cells that were unresponsive to the treatment will become the dominant cell type, which makes the tumor drug-resistant and more challenging to treat further. The term for this phenomenon is called selective pressure.[10]

A study observed 20 patients with TNBC treated with neoadjuvant chemotherapy (NAC) to combat highly heterogeneous cells, a characteristic of TNBC. They determined that this type of resistance can be acquired and adapted. Additionally, they found evidence that the patients who experienced relapses, as opposed to cancer elimination, had cells with genetic markers for that chemoresistance. The researchers were able to determine potential treatments for the cells that had genetic resistance using single-cell RNA and DNA sequencing. These treatments included EMT signaling, P13K inhibitors, and hypoxia inhibition using HIF-1 inhibitors.[47, 48]

Another study investigated the role of tumor heterogeneity in the resistance to EGFR-targeted treatment in colorectal cancer cells. Three cetuximab-resistant derivatives of LIM1215, OXCO-2, and DiFi cell lineages were utilized in next-generation sequencing, immunohistochemistry, and proliferation assays to identify the mechanisms of drug resistance in tumor cells. The results in the cell proliferation assays showed that colorectal cancer cells with developed resistance to cetuximab and panitumumab secrete transforming growth factor alpha (TGF-α) and amphiregulin. These secreted growth factors protect the encompassing sensitive cells from EGFR blockade by sustaining EGFR/ERK signaling in sensitive cells. The results showed that TGF-α and amphiregulin binding to EGFR caused a longer retention time of the receptor on the surface of the plasma membrane and redirected EGFR to the recycling pathway rather than to proteasomal degradation. This can potentially enhance the pro-proliferating effect of the protective microenvironment.[49]

3. Treatments of Interest

3.1 Immunotherapy

Cancer immunotherapy utilizes the body's immune system against cancer. Some patients possess immune system components that naturally fight the cancer cells, called tumor-infiltrating lymphocytes (TILs), while others do not. Despite a patient possessing TILs, the immune system still has difficulty fighting the cancer cells since they are, by definition, abnormal and lack certain processes that normal cells should have, such as missing proteins.[49] Immunotherapy includes various treatment options, including immune checkpoint inhibitors, adoptive cellular therapy (ACT), monoclonal antibodies, treatment vaccines, and immune system modulators.[50,51]

One type of immunotherapy that has shown great promise in recent years is chimeric antigen receptor (CAR)-T cell therapy, a type of ACT. Unfortunately, it is only approved for the treatment of blood cancers, and research into its effects on solid tumors is ongoing. CAR-T cell therapy uses either analogous or allogeneic donated T cells that can be found as part of the immune system. These cells are then genetically modified to express

CARs that target a specific antigen found on the surface of the intended cancer cells. This therapy is designed to boost the immune system and provide a specific target. Figure 2 summarizes the steps of CAR-T cell therapy. A disadvantage of this therapy is that it can lead to selective pressure since T cells only target one antigen at a time. Selective pressure refers to the eventual resistance of a heterogeneous tumor with multiple antigens to CAR-T cell therapy. This process is due to the elimination of all of the cells within the target antigen, leaving only the cancer cells that do not have the target antigen and therefore do not respond to the therapy.[51] Elimination of the target antigen is also known as antigen loss or escape.

Many ongoing clinical trials seek to determine if CAR-T cells are effective treatments for cancers with solid tumors, given that it is an approved treatment for blood cancers. Trials, even those without reliable results, are relevant to conversations about treatments of interest because it demonstrates the researchers' belief that these therapies have great potential to be effective. Until current research shows promising results, the success of the treatment will remain unknown.

One clinical trial run by Fred Hutchinson Cancer Center from 2016-2021 investigated the use of modified CAR-T cells to find ROR1 proteins on cancer cells from different cancer types, including but not limited to non-small cell lung cancer (NSCLC) and TNBC, both of which present as solid tumors. Participants with either NSCLC or TNBC had unsuccessfully undergone chemotherapy and other traditional treatments. Upon undergoing the clinical trial, all patients displayed adverse effects with mixed responses to the therapy. One out of three patients in dosage level 2 experienced either complete or partial remission, one out of six patients in dosage level 3 experienced progression-free survival after one year, and, across all dosage levels, there was a 38.89% overall survival rate.[52,53]

A second study funded by the National Natural Science Foundation of China and conducted at Nanjing Normal University analyzed ways to enhance CAR-T cell therapy. Knowing that TIGIT was a suppressor of anti-tumor processes and that MSLN was highly expressed in breast, prostate, and ovarian cancers, researchers combined an anti-α-TIGIT with MLSN CAR-T cells as a treatment. This combination significantly

enhanced the anti-tumor properties of MLSN CAR-T cells. The number of CAR-T cells positive for TIGIT was initially 18.3% but dropped to 1.81% after the addition of anti-α-TIGIT, which led to a more efficient MLSN CAR-T cell. [53] Additionally, a third Phase I trial is ongoing to treat prostate cancer with CAR-T cells modified for PSCA. The City of Hope Medical Center began this study in 2019 and it is estimated to complete in late 2023/early 2024. No current results are available.[53,55]

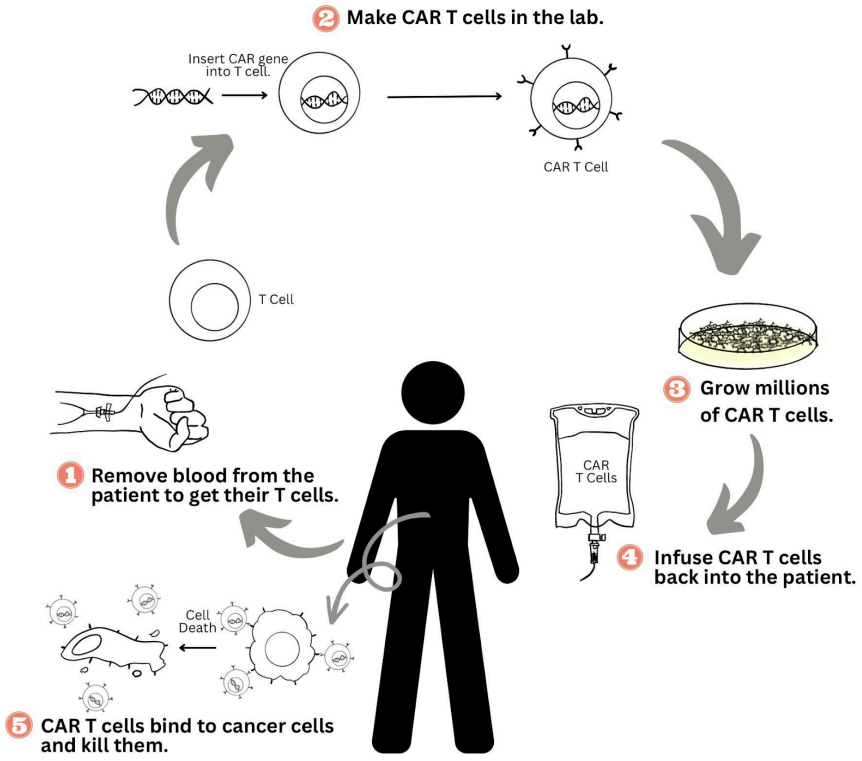

Figure 2. CAR-T cell therapy procedure. In CAR-T cell therapy, blood is first removed from the patient to obtain their T cells. CAR-T cells are then engineered and grown in the laboratory before being infused back into the patient. In the patient, the CAR-T cells would target and bind to specific antigens present on the tumor cells, killing them. In the long term, however, the tumor could acquire resistance to this therapy through antigen loss.

A more common type of immunotherapy treatment is immune checkpoint inhibitors (ICIs). These checkpoints are part of the immune system and prevent immune cells from reacting too aggressively and attacking beneficial cells.[50] However, these checkpoints can also prevent the immune system from effectively dealing with cancer cells. Hence, by blocking these checkpoints, scientists are able to permit the immune system to start

treating the cancer cells fully. The most common immune checkpoints targeted are cytotoxic-T-lymphocytes-associated proteins (CTLA-4), programmed cell death 1 (PD-1), and programmed cell death ligand 1 (PD-L1).[56] Figure 3 shows the mechanism of this checkpoint blockade.

There are a variety of clinical trials that involve ICIs. Many of them use combination therapy by combining one of the seven FDA-approved ICI treatments with either another approved ICI treatment or with another therapy like chemotherapy.[56] A clinical trial run by Bristol-Myers Squibb from 2016-2023 tested the combination of ipilimumab and nivolumab (treatment A, immunotherapy) against pemetrexed and cisplatin or carboplatin (treatment B, type of chemotherapy) in malignant pleural mesothelioma (MPM). Treatment A had an overall survival that was, on average, four months longer than treatment B. The median disease control rates for treatment A and treatment B were 76.6% and 85.1%, respectively.[57] In a three-year minimum follow-up, the trial showed overall survival rates of 23% and 15% for treatment A and treatment B respectively. Moreover, at three years, 28% of patients had an ongoing response to treatment A while treatment B had 0% patients with ongoing response.[58] These results demonstrate how the combination of nivolumab and ipilimumab continued to provide long-term survival benefit over chemotherapy, supporting this combination of ICIs as a first-line treatment for unresectable MPM.

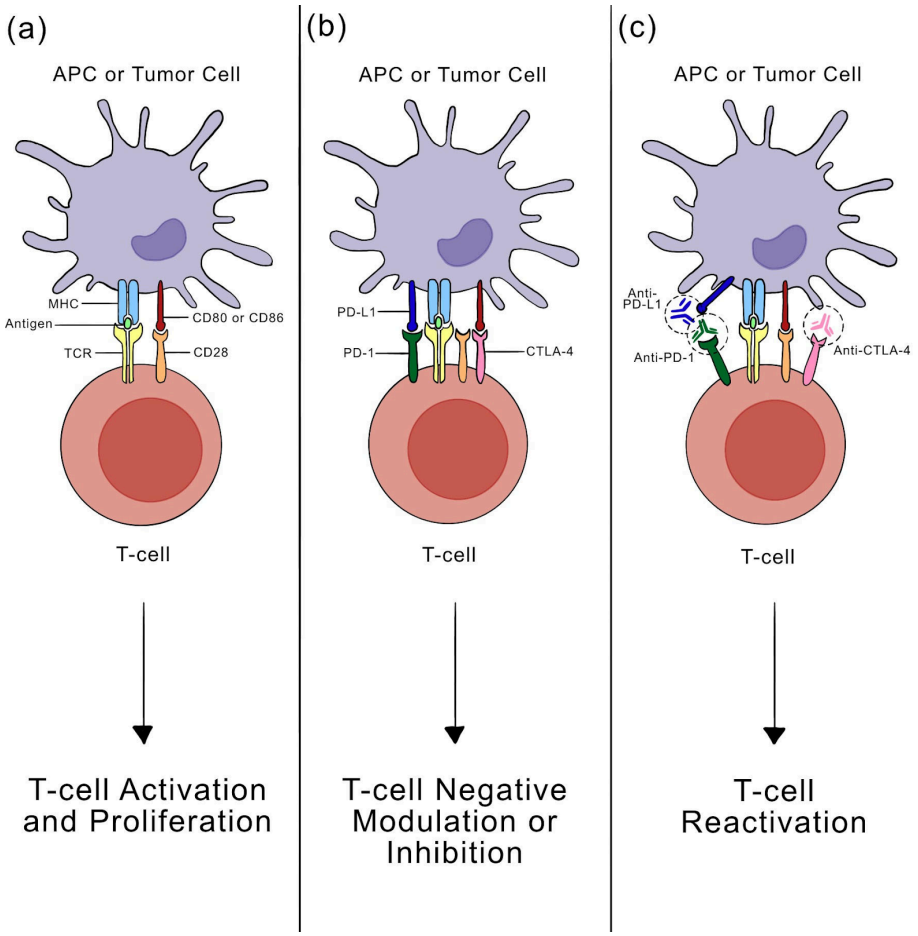

Figure 3. Mechanism of immune checkpoint inhibitors. (a) An antigen-presenting cell (APC) displays an antigen bound by the major histocompatibility complex (MHC). T cell receptors (TCR) recognize the antigen, causing interaction between the T cell and the APC or tumor cell. A co-stimulatory signal caused by CD80/86 binding to CD28 results in T cell activation and proliferation, allowing the T cell to kill the cancer cell. (b) When CTLA-4 is present on a T cell, it binds to CD80/86 in place of CD28. PD-1 on T cells also bind to PD-L1 that are present on tumor cells. Both of these interactions lead to an inhibitory signal that blocks the T cell from killing the cancer cell. (c) Anti-CTLA-4 binds to CTLA-4, blocking its interaction with CD80/86. This allows for binding of CD28 to CD80/86, producing a co-stimulatory signal. Similarly, anti-PD-1 binds to PD-1 while anti-PD-L1 binds to PD-L1, blocking PD-1 and PD-L1 interactions. Thus, through immune checkpoint inhibition, T cells are reactivated and can kill tumor cells.

3.2 Combination Therapy

Combination therapy involves using multiple types of treatment. It can be a combination of drugs, immunotherapies, chemotherapy, radiation, and other cancer treatments. Since cells respond to different therapies, a highly heterogeneous tumor likely needs multiple treatments in order to eliminate

all tumor cells from the body. However, too many drugs can strain the body and be highly toxic. Combination therapy thus seeks to maximize efficiency while minimizing toxicity. By using existing treatments, it can also be cheaper and more time-effective to research than developing a new drug or treatment.[59]

One study on mice, published in 2018 and funded by the Canadian Cancer Society, used oncolytic viruses and the HDAC inhibitor MS-275 to prevent relapse from ACT. The CD8+ T cells targeted a specific antigen on the tumor. With only ACT, there was a significant improvement in tumor size; however, selective pressure had occurred until only antigen-negative tumor cells were remaining, signifying that the T cells could no longer target those tumor cells. The use of oncolytic viruses and MS-275 was shown to prevent any relapse and change tumor-infiltrating myeloid cells into pro-inflammatory cells, allowing for better recovery.[60] In a Phase II clinical trial funded by Bristol Myers Squibb that ended in 2018, researchers found that patients with melanoma responded favorably to a combination of melphalan (chemotherapy) and the approved CTLA-4 blocker ipilimumab (immunotherapy). Based on the data provided, 85% of patients had observable responses to the treatment, and there was a 58% progression-free survival (PFS) rate after one year, with no increase in toxicity at the site of the treatment.[61]

An ongoing clinical trial (2018-2024 estimate) by MedImmune LLC is testing various dosages of oleclumab and osimertinib in the treatment of NSCLC. The percentage of patients with disease control (complete response, partial response, or stable) and overall survival for each dose is as follows:

oleclumab 1 + osimertinib 1: 80%, 21.9 months

oleclumab 2 + osimertinib 1: 81%, 24.8 months

None of the patients that received the above doses had any dose-limiting toxicities within twenty-eight days of the first treatment. However, within ninety days of the last dose, all patients had adverse effects, such as infections and infestations like cystitis and pneumonia, as well as nervous system disorders like cerebral infarction and spinal cord compression. Even so, only

one patient who received the oleclumab 1 + osimertinib 1 doses had abnormal vital signs that were considered severe.[62] This shows the importance of finding the right amount of dose to give to the patient, especially in the case of combination therapy.

3.3 Dual/Multi Ex vivo Armed T Cells (EATs)

Dual- or multi-EATs are T cells armed with two or more bispecific antibodies (BsAbs). Generally, T-cell-engaging bispecific antibodies (T-BsAbs) bind specifically to a tumor-associated antigen (TAA) and a CD3 subunit that forms a complex with the tumor cell receptor (TCR). T-BsAbs can thus link tumor cells and T cells together, activating T cells and leading to tumor death. Moreover, CD3 engagement stimulates the T cells' immune response, which redirects host immunity toward tumors. Hence, T-BsAbs are a promising antibody therapy for various cancers.[64] A Phase I/II clinical trial was conducted on epcoritamab, a T-BsAb that targets CD3 and CD20. This targeting redirects and activates T cells to kill CD20-expressing malignant cells in relapsed or refractory Large B-Cell Lymphoma (LBCL). Among 157 patients, the overall response rate (ORR), defined as the proportion of patients who have a partial or complete response to therapy, was found to be 63.1%.[64] An ORR value greater than 60% is Grade 3 and is considered a high value, showing the high efficacy of epcoritamab.[65] The complete response (CR) rate was 38.9%, with the median time to CR being 2.7 months.

Responses with epcoritamab were also shown to have transitioned from partial response (PR) to CR at the later assessments in nine patients. These results suggest there is an added benefit in certain patients with continuous treatment using this T-BsAb.[64] Another clinical trial found that ABBV-383, a B-cell maturation antigen x CD3 T-BsAb, could treat patients with relapsed or refractory multiple myeloma with an ORR of 68% at ≥ 40 mg dosage, showing the T-BsAb's promise in treating already heavily-treated patients at that dosage amount.[66]

EATs are similar to T-BsAbs in that they are also able to crosslink tumor cells and T cells together, activating the subsequent immune response. However, in EATs, the BsAbs are already attached to the T cells, which makes them similar to CAR-T cell therapy. The only difference is that they

are armed with BsAbs instead of CARs. With multiple BsAbs attached, dual- or multi-EATs can target a wider variety of TAAs, thus helping to overcome tumor heterogeneity.

A study by Park and Cheung tested the efficacy of dual-antigen targeting strategies using different kinds of EATs, including pooled-EATs (EATs with unique specificity administered simultaneously), alternate-EATs (EATs with unique specificity administered in an alternating schedule), dual-EATs, TriAb-EATs (T cells armed with a BsAb specific for two targets besides CD3), and multi-EATs, with GD2 and HER2 as target antigens. Among these, they found that dual- and multi-EATs had the most potential in overcoming tumor heterogeneity and target antigen loss, both of which are challenges to current T cell immunotherapies. Dual-EATs, armed with GD2- and HER2-BsAbs, and multi-EATs, armed with GD2-, HER2-, CD33-, PSMA-, and STEAP1-BsAbs, had induced stronger cytotoxicity against a mixed lineage of cancer cells than mono-EATs armed with only one type of BsAb. This stronger cytotoxicity resulted in a more potent anti-tumor response and dual- and multi-EATs exceeded the efficacy of mono-EATs, significantly improving tumor-free survival. They, along with alternate-EATs, were also successful in inducing tumor regression, giving rise to long-term survival. Furthermore, dual- and multi-EATs exerted a synergistic anti-tumor effect when they encountered multiple antigens simultaneously, which played a significant role in preventing antigen loss.[67]

However, experiments have only been done in mouse models. Even though no additional toxicities that could cause serious or fatal effects upon infusion of CAR-T cells or BsAbs were observed, the same results might not be reproduced in humans. The BsAbs also hold specificity for human antigens, not mouse antigens, so using a mouse model fails to mimic human diseases and their therapeutics perfectly. Although the T cells, tumors, and BsAbs were of human origin, the tumor microenvironment contained cells of mouse origin, which included tumor-infiltrating myeloid cells, fibroblasts, vasculature, and lymphatics. These could interact with one another and affect tumorigenesis and anti-tumor response. Despite these limitations, dual- and multi-EATs have potential to overcome tumor heterogeneity and cancer resistance.[67] Moreover, they could potentially be

used in more targeted and personalized treatments by arming the T cells with BsAbs that target specific TAAs found in a patient's tumor.

3.4 NanoRNPs with a Combination of Single Guide RNAs (sgRNAs)

In the early stages of integrating nanotechnology into cancer therapy, profound strides were made in improving existing therapies. One such advancement has been the development of nanotechnology-mediated drug delivery systems to enhance their delivery to tumor sites and reduce systemic toxicity.[68] Meanwhile, nano-Cas9 ribonucleoprotein (nanoRNP) with a combination of sgRNAs harnesses the full potency of nanotechnology and gene editing for precision treatment.

In a study by Liu et al., a nanoRNP system that could carry any combination of sgRNAs was demonstrated to achieve targeted gene disruption and effective suppression of heterogeneous tumors. NanoRNP has a core-shell structure linked by CA that degrades under acidic conditions. This way, it maintains a stable structure in blood circulation and normal organs but detaches its shell when in the acidic tumor microenvironment. This action facilitates tumor accumulation, cell internalization, and eventual gene editing by the Cas9/sgRNA complex in its core. With Cas9, nanoRNPs can disrupt the targeted gene sequence under acidic conditions, significantly downregulating the expression of the target genes. When nanoRNPs carry a combination of sgRNAs, they simultaneously disrupt the expression of multiple target genes, and this could potentially overcome the genetic heterogeneity that causes treatment resistance in cancer.[69]

In a heterogeneous tumor model at pH 6.5, Liu et al. expressed the target genes STAT3, which increases tumor cell proliferation, survival, and invasion while suppressing immunity towards tumors, and RUNX1, whose increased levels correlate with cancer cell proliferation, tumoral angiogenesis, and metastasis. The nanoRNP carrying a combination of sgRNAs, nanoRNP-STAT3+RUNX1, disrupted the expression of both genes, inhibiting the proliferation of the tumor cells. It also increasingly induced cell apoptosis in the tumor. In contrast, nanoRNPs carrying a

single type of sgRNA led to the reduced expression of only one of the genes, resulting in partial growth inhibition in the heterogeneous tumor. However, through an analysis of gene disruption on STAT3 and RUNX1, it was discovered that complete reduction of the target genes could not be achieved by the nanoRNP, even if it carried multiple different sgRNAs. Even so, nanoRNPs carrying a combination of sgRNAs could simultaneously suppress the proliferation of multiple tumor cell subpopulations, showing their potential to overcome tumor heterogeneity.[69] Similar to dual- or multi-EATs, this treatment strategy can be used in a more personalized approach, wherein the nanoRNPs could carry the sgRNAs required to disrupt the specific target genes expressed in a patient's tumor.

3.5 Circulating Tumor Cells (CTCs)

CTCs, rather than a treatment, are better described as a research methodology. They are cells in the blood that come from a tumor, and they have the potential to become the primary way to test and develop new drugs, as well as to test for the progression of cancer. More traditional methods have various problems. For instance, 2D cultures lack the complexity of a tumor structurally on the genetic and physical level but can be tested in high volumes at high speeds. Patient-derived xenografts fix the structural problems of 2D cultures, but they are unable to be used in high-throughput screenings, which significantly reduces research speed. CTCs maintain the level of heterogeneity and the tumor's structure, which allows for high-throughput screenings. These advantages allow for new and more personalized treatments as these therapies can be tested on an accurate model that poses zero risk for the patient.[70]

The greatest challenge to utilizing CTCs is accurately isolating them from other cells in the blood since CTCs are very rare in the blood and little is known about their genetic structure.[71,72] CTCs can be isolated based on differences in their physical properties, such as density, size, deformability, and electrical properties. However, these methods are very inefficient as they lack purity and specificity. For this reason, researchers usually use CTC-related technologies based on biological properties, particularly

techniques dependent on the epithelial cell adhesion molecule (EpCAM), a marker positively enriched in CTCs.[72]

CTCs are also indicators of prognosis and a non-invasive method of determining whether a treatment is working. Through liquid biopsies[70], doctors can determine the CTC count in a patient's blood.[70] In a study that analyzed blood samples from 59 patients with esophageal squamous cell carcinoma, CTC levels were found to be correlated with overall survival (OS) and PFS rates. Researchers found that the overall and progression-free survival rates were significantly better for patients with a CTC count of less than three. The mortality rates for the patients with either >0, >5, and >7 CTCs per 7.5 mL were 65.2%, 78.4%, and 87.5% respectively.[73] A lower CTC count after treatment is also associated with an excellent prognosis.[71,72] There are also limited studies that suggest CTCs can be used for early cancer detection, although this has only been shown in mouse models.[71]

4. Future Directions

Dual- or multi-EATs and nanoRNPs with a combination of sgRNAs have strong potential to confront therapeutic resistance, making them promising improved treatment strategies for cancer. These strategies would also provide more insight into the design of more advanced and effective cancer therapies. However, they have only been studied in mouse models and heterogeneous tumor models respectively, both of which may not represent a human system perfectly. Hence, before these treatments can undergo clinical trials and be used as personalized approaches for tumor heterogeneity, more research must be conducted to ensure that side effects, such as off-target toxicities, are minimized.[67,69] On another note, further research on optimizing models to better imitate human diseases would undoubtedly be useful for preclinical testing of the safety and efficacy of drugs and treatment strategies.

CTCs, in turn, are promising as a way to determine the progress and clinical efficiency of a treatment. The CTC count can be used as an indicator that a treatment is no longer effective, triggering a change in the type of therapy a patient is receiving. Since a key challenge is the rarity of CTCs, more research has to be done to find ways to identify and isolate CTCs. This

could then contribute to a larger sample size that can be used in studies, improving their reliability.[70]

The emerging technologies and methodologies for assessing tumor heterogeneity are clear implications of the advancement of personalized treatment and precision targeting for heterogeneous tumors. Currently, novel targets and therapeutic strategies for overcoming tumor heterogeneity are critical in cancer research. Cancer research is extremely complicated and unique because each type of cancer for every individual is different, one factor of which is tumor heterogeneity. This poses a major barrier to producing a generalized approach and treatment for all individuals suffering from this disease. Because tumors possess the capability to overcome therapeutics and treatments, scientists and medical researchers are constantly faced with new challenges in producing a cure for cancer. On the other hand, novel techniques that analyze individual cells and their distribution, such as single-cell sequencing and spatial transcriptomics, have transformed our predictive value and comprehension of the complex cellular organization.[74,75] All of the mentioned novel technologies and ongoing clinical trials pave a clearer path toward finding a more target-specific treatment for cancer. Once tumor heterogeneity is addressed, research can focus on other complex aspects of cancer treatment.

5. Conclusion

Tumor heterogeneity is the largest barrier in developing ground-breaking and target-specific cancer treatments. With therapeutic resistance forming among all forms of cancer, tumor heterogeneity highlights the need for novel treatments that will overcome this barrier. Current therapies that can limit cancer disease progression include immunotherapy, particularly CAR-T cell therapy, and combination therapy. There are also novel strategies, such as dual- or multi-EATs, nanoRNPs with a combination of sgRNAs, and CTCs. Each strategy presents their own respective challenges that limit them to mouse model studies or insufficient target gene reduction. Ongoing clinical trials exist to reduce such limitations and challenges and discover crucial information for the treatment of humans. Since no single treatment has yet been discovered to completely overcome

tumor heterogeneity, medical research in developing personalized treatment and precision targeting is thus vital in saving lives.

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