Heterogeneity and individualized treatment of microenvironment in glioblastoma (Review)

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Abstract. The heterogeneity of glioblastoma can suppress immune cell function and lead to immune evasion, which presents a challenge in developing effective molecular therapies for tumor cells. However, the study of tumor immune heterogeneity holds great potential for clinical immunotherapy. Liquid biopsy is a useful tool for accurately monitoring dynamic changes in tumor immune heterogeneity and the tumor microenvironment. This paper explores the heterogeneity of glioblastoma and the immune microenvironment, providing a therapeutic basis for individualized treatment. Using liquid biopsy technology as a new diagnostic method, innovative treatment strategies may be implemented for patients with glioblastoma to improve their outcomes.

Contents

1. Introduction

Glioblastoma (GBM) is characterized by significant genetic heterogeneity among tumor cells. This heterogeneity is referred to as 'polymorphism', as tumor cells rapidly undergo mitosis, resulting in the formation of numerous subclones and uncertainty regarding the state of the genome. Understanding the heterogeneity of GBM depends on the location of sampling and analysis of subclonal fractionation or indeterminate genomic status. Tumor cells are influenced by both genetic factors and environmental elements in the microenvironment, resulting in a complex regulation process (1). In addition, intrinsic differences in subclonal tumor cells that arise from random mutations can create distinct niches within limited lesions. These cells are then forced to compete for growth and nutrients (2). Various subclonal tumor cells have the ability to modify the tumor microenvironment in order to obtain adaptations. These adaptations include inducing angiogenesis to acquire nutrient supply, interfering with immune stimulation/inhibition checkpoint pathways to promote immune evasion and remodeling the extracellular matrix to promote metastasis (3). However, rather than actively modifying the environment, multiple mechanisms guide the evolution of tumor cells through the selection of subclones with the most adaptive phenotype by environmental factors (1). Multiregional whole-exome or genome sequencing has revealed that there is significant variation in the genetic makeup of tumor cells across different anatomical locations and within the same tumor over time (4). Furthermore, tumor heterogeneity has a significant impact on both the immune microenvironment and the infiltration of various immune cells within tumors, such as cytotoxic T lymphocytes (CTLs) (5), myeloid antigen-presenting cells (6) and cancer-associated fibroblasts (CAFs) (7). This heterogeneity can vary greatly between different types of immune cells, leading to further complexity in understanding the immune response to tumors. It has been discovered that the genetic structure of tumor cells and the components of the immune microenvironment interact with each other. This interaction results in a more complex alteration of both the heterogeneity of tumor cells and the heterogeneity of the tumor microenvironment. Consequently, the heterogeneity of tumor cells is constantly evolving. It has been discovered that the genetic structure of tumor cells and the components of the immune microenvironment interact with each other. This interaction results in a more complex alteration of both the heterogeneity of tumor cells and the

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heterogeneity of the tumor microenvironment. Consequently, the heterogeneity of tumor cells is constantly evolving (8). Thus, tumor heterogeneity has an important role in tumor development.

2. Origins of immune microenvironment heterogeneity

High-throughput sequencing methods are utilized to analyze the mutational spectrum and evolutionary trajectories of tumor cells. These studies reveal a wide range of genetic tumor heterogeneity in both spatial and temporal dimensions, encompassing diverse single-nucleotide mutations, insertions, deletions and copy number variations (9,10). In primary tumors, mutations in driver genes frequently provide a survival advantage and give rise to a dominant clonal population. By contrast, mutations in noncoding regions do not provide significant growth advantages during tumor evolution (11). Therefore, tumor evolution and spatiotemporal heterogeneity are driven by genetic instability originating from both clonal and subclonal tumor cells. This genetic heterogeneity also shapes the antigenic profile of the tumor, ultimately contributing to the heterogeneity of the tumor immune microenvironment (12). Neoantigens, which are primarily derived from non-synonymous mutations and insertions/deletions, were found to be the primary drivers of $CD8^+$T cell-specific differences. This suggests that genetic instability is the root cause of the heterogeneity observed in the immune microenvironment. Neoantigens have a significant role in the variation of $CD8^+$T cell specificity, highlighting the importance of genetic instability in the diversity of the immune microenvironment. Studies have shown that neutrophils, macrophage M2 polarization and the inflammatory index are associated with worse prognosis and overall survival in glioblastoma (13-15). These findings indirectly suggest the presence of an inflammatory response in tumor cells, as well as a deficiency in the mechanisms that can trigger an immune response.

Epigenetic modification. The formation of heterogeneity in the tumor immune microenvironment is shaped by epigenetic remodeling of tumor cells, as evidenced by various studies (16). This remodeling primarily occurs through alterations in DNA modification, chromatin accessibility and post-transcriptional regulation, such as gene expression mediated by non-coding RNA interference. Epigenetic modifications have a crucial role in accelerating the malignant transformation of tumor cells and influencing the tumor immune microenvironment (17). Various mechanisms, including methylation, chromatin instabilities and epigenetic remodeling, provide adaptive advantages to tumor cells in response to their environment, such as in the progression of lung cancer *in situ* (16). These epigenetic modifications result in marked heterogeneity in both spatial and longitudinal dimensions of genetic progeny, and also impact tumor progression and immunogenicity by altering chromatin accessibility and expression through immune-related elements (18). Epigenetic modifications have been linked to high levels of heterogeneity in various tumor types, such as acute myeloid leukemia and glioblastoma, similar to genetic instability.

Adaptability of the microenvironment. Tumor cells and immune components in the microenvironment are constantly exposed to radiation and chemotherapy, leading to adaptive mechanisms in both tumor and immune cells to establish a new balance (19). This balance is affected by the intrinsic heterogeneity in tumor cell driver mutations or molecular signatures, resulting in varying responses to therapy. In cases where localized tumor clones fail to survive treatment, they release large amounts of ATP through autophagy-mediated cell death (20). ATPs have the ability to promote chemotaxis and generate an inflammatory response in tumors. However, overcoming immune heterogeneity has been shown to be associated with therapeutic resistance and radio-resistance (21). On the other hand, the presence of extracellular nucleases can rapidly digest ATPs to adenosine in the extracellular matrix, creating an inhibitory immune microenvironment. This can hinder the immune response. In addition, T-cell phenotypes can change significantly in response to immune checkpoint blockade, affecting the immune cells. The immune response to cancer treatment is characterized by a change of T-cell subsets and cytokine production (22). For instance, certain patients with glioblastoma who undergo chemotherapy exhibit a marked increase in $CD8^+$T cell proliferation post-treatment. The intricate and ever-changing interplay between drug therapy, tumor cells and immune cells has a crucial role in shaping the heterogeneous immune microenvironment over time and space.

Microenvironment heterogeneity in tumor immune components. The tumor immune microenvironment is primarily characterized by two components: Tumor and non-tumor. These components are spatially distinct and have different localization and abundance/activity. For instance, the inhibitory immune checkpoint programmed cell death 1 (PD-1) ligand 1 is expressed on the surface of tumor cells (23), while immunosuppressive or pro-inflammatory cytokines are secreted by both tumor and non-tumor cells (24,25). Inhibitory or effector cells can infiltrate the microenvironment and the state of the vasculature can also impact the immune response (26). In addition, the spatial distance of the marginal region and the distribution of metabolized nutrients has a role in the microenvironment (27,28). The spatial variations discussed in a subject paper have a significant impact on both clinical prognosis and treatment response (29). Various tumor types have been found to exhibit intertumoral heterogeneity of immune cells beyond T-cell subsets (30). For instance, in glioma, macrophages with CD44 and CD109 phenotypes were predominantly situated in the stroma, while the expression of soluble CD10 was abnormally elevated in the core area compared to the edge area (31).

Across different phenotypes, there is heterogeneity in T-cell clonality, proliferative potential, differentiation stage, functional polarization, cytokine secretion profile and metabolic environment. In terms of T-cell repertoire propensity, expanded/proliferated T-cell receptors (TCRs) are further categorized as common TCR clones (which are detected in all regions within the tumor) or regional clones (which have a heterogeneous distribution) (30). The burden of common and regional nonsynonymous mutations was found to be positively correlated with the number of common and regional TCR

clones, suggesting regional heterogeneity and antigen-driven T-cell proliferation. In addition, the metabolic profile has been identified as a crucial regulator of the immune microenvironment, potentially influencing the proliferation potential and adaptability of cancer cells to their surroundings (32). The proliferation potential and adaptability of cancer cells in the surrounding environment are affected by metabolic characteristics, resulting in the production of immunosuppressive mediators such as lactate and adenosine. These mediators reduce the effectiveness of cytotoxic immune surveillance. Cells with high glycolytic activity and malignant cells can switch their metabolic pathways to anabolic reactions, which can impact their behavior (33).

Tumor and immune cells can be influenced by both genetic and non-genetic environmental factors, which can ultimately impact the progression of the disease and the response to antitumor therapy. In addition, these factors can also affect the dynamics of the tumor cells themselves (34,35). For instance, RNA-Seq analysis conducted on patients with pancreatic ductal adenocarcinoma demonstrated significant alterations in the composition of immune cell infiltration as the disease progressed from a non-invasive lesion to an invasive phenotype (36). Disease progression across multiple tumor types is typically characterized by a decrease in infiltration of CD8+T cells and dendritic cells, as well as an abnormal accumulation or expansion of immunosuppressive cells such as T-regulatory cells, myeloid-derived suppressor cells or CAFs. In addition, impaired cytolytic activity, expansion of the cell repertoire, clonality restriction and progressive T- and B-cell exhaustion are also commonly observed. Furthermore, spatiotemporal heterogeneity has been observed in different tumor types, such as lung cancer (37), melanoma (38) and patients with intracranial metastatic disease (39). The significance of this heterogeneity for disease prognosis is reinforced by the inverse relationship between the presence of immune-favorable regions or lesions in individual patients and disease control and survival outcomes (40). The present review focuses on new theragnostic and therapeutic approaches that can combat the heterogeneity of the tumor microenvironment (Fig. 1; Table I).

3. Liquid biopsy

Recent studies have demonstrated the potential of identifying extracellular vesicles, DNA and RNA particles, which are collectively known as 'liquid biopsies', in monitoring disease progression. This approach offers a glimpse into the heterogeneity of the entire tumor cell population. Liquid biopsy is a quick and cost-effective method for obtaining tumor-related information. Integrating the analysis of extracellular vesicles and related circulating nucleic acids into clinical practice can aid in establishing a non-invasive diagnosis, conducting complex tumor therapy and monitoring disease progression throughout the clinical course (41). There is currently no agreement among researchers regarding the optimal nucleic acid types, biological fluids or pre-analysis/analysis techniques for obtaining the most accurate results (42,43). However, high-throughput sequencing technology, nanopore sequencing technology and digital drop PCR have shown promise in analyzing the genome and detecting various circulating DNA types, such as cell-free DNA (cfDNA), circulating tumor DNA (ctDNA) and mitochondrial DNA (mtDNA). These technologies can potentially enhance the application of liquid biopsy technology. cfDNA is a double-stranded structure that is usually 150-200 base pairs long and has a concentration of ~10-15 ng/l. In patients with cancer, the level of cfDNA increases significantly and the composition of cfDNA changes over time. It is important to note that ctDNA, which originates from tumor cells, is the major fraction of cfDNA (44). The detection of mutation-carrying ctDNA in plasma provides valuable information about genetic changes in tumor tissue. However, ctDNA levels are typically very low and have a short half-life in the case of GBM. However, with the aid of highly sensitive and tumor-specific NGS, mutations in B-RAF proto-oncogene, serine/threonine protein kinase, isocitrate dehydrogenase (IDH1) and IDH2, and amplification of tyrosine kinase receptor 2, mesenchymal-epithelial transition factor, EGFR and platelet-derived growth factor receptor (PDGFR) α, have been identified. In cases of primary brain malignancies, high levels of ctDNA can be detected in cerebrospinal fluid (45). Changes in mtDNA can occur at an early precancerous stage and elevated levels of mtDNA are indicative of a poor prognosis. In addition, mtDNA may be present in the future and is crucial in the early detection of new-onset and recurrent GBM (46) (Fig. 2).

4. Treatment approaches

The objective of identifying new therapeutic targets is to hinder various signaling pathways and stop the growth of drug-resistant subclones, as well as immunosuppressive effects (47). To date, the Food and Drug Administration has approved two antineoplastic agents, temozolomide and bevacizumab, and Tumor Treating Fields therapy for GBM after postoperative radiotherapy and chemotherapy for glioblastoma (48). In the BELOB study, the combination of bevacizumab and lomustine was proven to significantly enhance the overall survival rate of patients who suffer from recurrent GBM (49). Furthermore, the effectiveness of neoadjuvant pembrolizumab therapy has been demonstrated in cases of recurrent and operable glioblastoma (50). Glioblastoma is characterized by an immunosuppressive tumor microenvironment, minimal antigen presentation, unique antigen escape mechanisms and direct immunosuppression (51). Overall patient survival may be increased through an individualized approach that combines drugs and various treatments (Table I).

Immune checkpoint inhibitors (ICIs). Tumor cells can evade cellular immune responses by binding specific antigens expressed on the surface of T cells, such as CTL-associated protein (CTLA)-4 and PD-1, to their corresponding ligands on the tumor surface. This results in reduced activation of cytotoxic T cells, which may lead to the tumor cells avoiding elimination. However, monoclonal antibodies that selectively block these antigens have been found to stimulate an immune response. According to clinical trials, anti-CTLA4 (nivolumab, pilimumab) and anti-PD1 (pembrolizumab) have shown promising results (51). In addition, the enzyme indoleamine 2,3-dioxygenase, which has a crucial role in T-cell activity, has exhibited checkpoint activity inhibition through the use of methylated tryptophan indole oxime.

Table I. Associated clinical trials for immunotherapy of GBM.

Strategy	Regimen	Condition	Trial ID
Immune checkpoint inhibitors	TIM-3 and LAG-3 vs. PD-1	GBM	NCT02658981
	Anti-PD-1	GBM	NCT02017717
Vaccine therapy	EGFRvIII	GBM	NCT01967758
	Peptide vaccine	IDH1-mutated grade III and IV gliomas	NCT02193347
	Dendritic cells	GBM	NCT00045968/ NCT02146066
	GAPVAC-101	GBM	NCT02149225
Oncolytic virotherapy	HSV-1rQNestin34.5v.2 + Cyclophosphamide	GBM	NCT03152318
	Polio-Rhinovirus Chimera (PVSRIPO)	GBM	NCT02986178
CAR-T and TCR-T treatment			
CAR-T	CD19	B-cell acute lymphoblastic leukemia	NCT02435849
TCR-T	CAR-T-EGFRvIII	GBM	NCT02209376
		Neuroblastoma	NCT01430390
Signaling pathway-focused therapy	Everolimus targeting mTOR	GBM	NCT04135807
Epigenetic therapy			
HDAC inhibitors	Valproic acid and temozolomide	GBM	NCT01204450
	Vorinostat and isotretinoin	GBM	NCT00217412
Radiosensitizer	L19TNF + Temozolomide	GBM	NCT04443010
Novel radiotherapy approaches	Targeted photodynamic therapy	GBM	NCT04469699
	Focal laser ablation	GBM	NCT05296122
	NovoTTF-100A + Temozolomide	GBM	NCT00916409

GBM, glioblastoma; PD-1, programmed cell death 1; PD-L1, PD-1 ligand 1; PVSRIPO, polio-rhinovirus chimera; HDAC, histone deacetylases; EGFRvIII, epidermal growth factor receptor variant III; TIM-3, T-cell immunoglobulin-3; LAG-3, CD223 lymphocyte activation gene 3.

Vaccine-based treatment. Vaccine therapy is an innovative approach to treating the immune system, and it has the potential to be a valuable tool in the battle against tumors. When tumor-associated and tumor-specific antigens are introduced, they stimulate an immune response. For instance, a protein vaccine called lindopipate, which targets EGFR variant III, has shown promising early results (52). One potential target for cancer treatment is survivin, a member of the inhibitor of apoptosis protein family, which can be targeted through the use of a protein-based vaccine called SurVaxM (53). Another effective approach is the use of activated autologous dendritic cells, such as DCVax. The most prominent personalized cancer vaccine is heat shock protein (HSP) peptide complex-96, which works by training the patient's immune system to recognize the gp96 HSP and its associated proteins. This protein is extracted from the patient's own tumor tissue (54).

Viral therapy. The use of viral vectors that can integrate into the host tumor genome is a form of immunotherapy. These vectors carry genes that code for enzymes or other proteins that can be lethal to tumor cells. Toca FC is a prodrug of the cytosine deaminase gene and 5-fluorouracil, which can cross the blood-brain barrier and be converted to 5-fluorouracil locally (55). Oncolytic viruses are viral particles that have been genetically engineered to target and destroy tumor cells, while leaving surrounding brain tissue unharmed. Delta-24-RGD has been shown to have a strong antitumor effect on its own and has also been effective when used in combination with pembrolizumab (56). Due to the variable infectivity of the virus, differential changes in treatment may occur. Consequently, viral therapy should be utilized as a complementary treatment rather than as a standalone option.

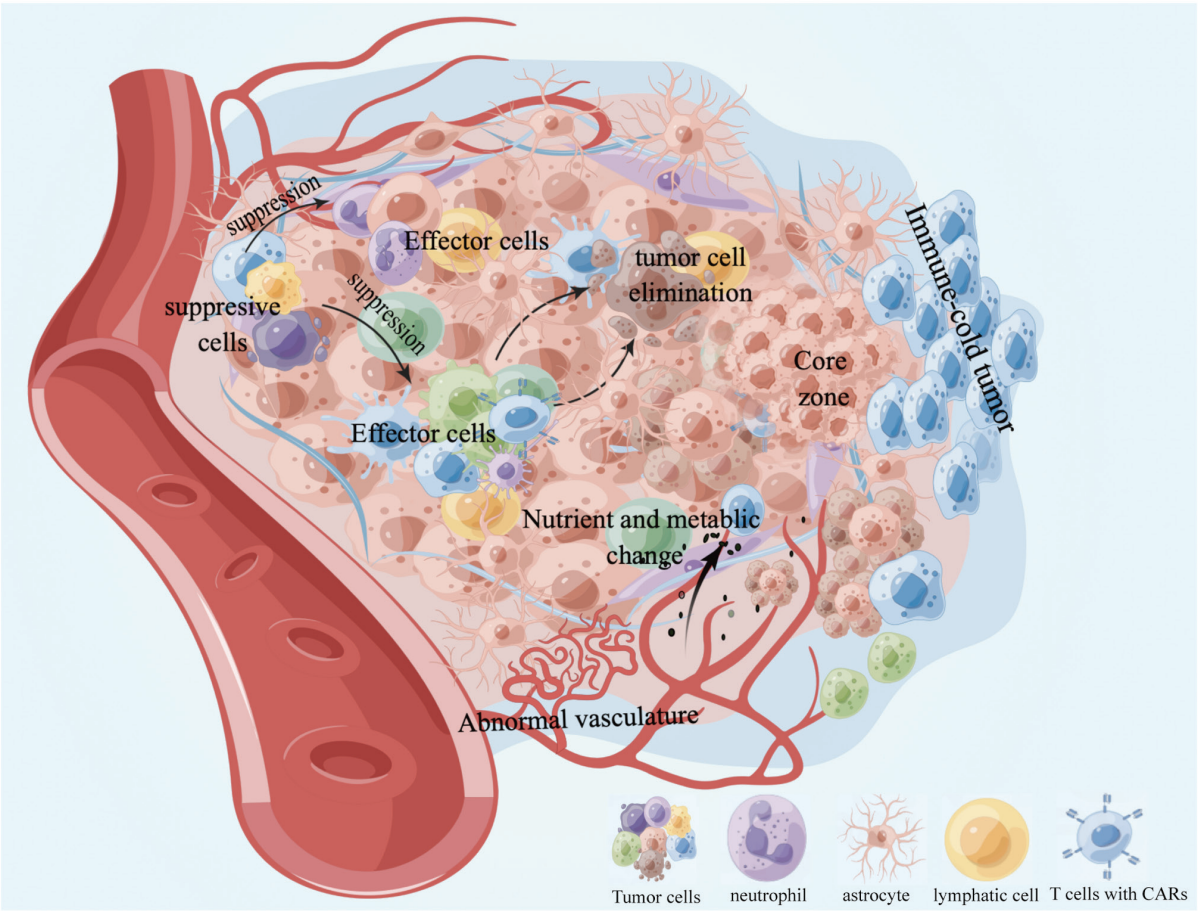

Figure 1. Tumor immune microenvironment. Representative features of heterogeneity in the immune microenvironment include novel antigenic profiles, immune suppression and effector cell infiltration, vascular system status, cytokines and metabolic processes. CAR, chimeric antigen receptor.

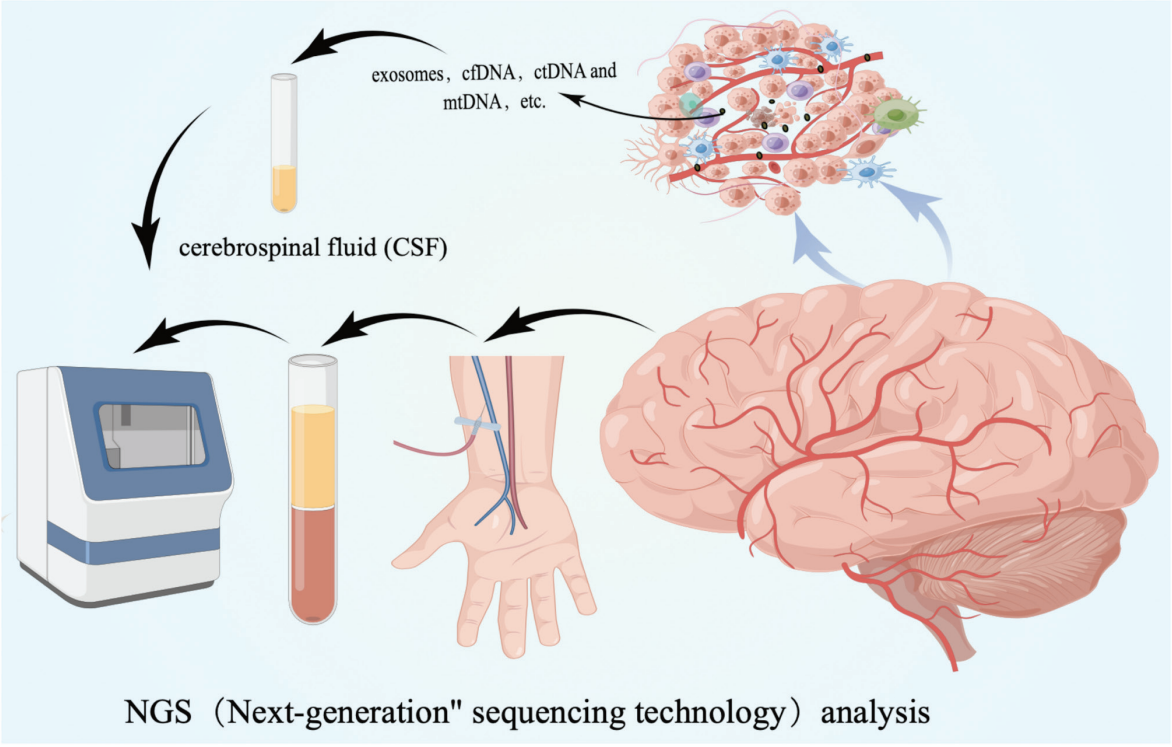

Figure 2. Glioblastoma-produced cfDNA, ctDNA and mtDNA, detected by NGS monitoring in blood or cerebrospinal fluid, may provide a personalized plan for developing different treatment strategies. mtDNA, mitochondrial DNA; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; NGS, next-generation sequencing.

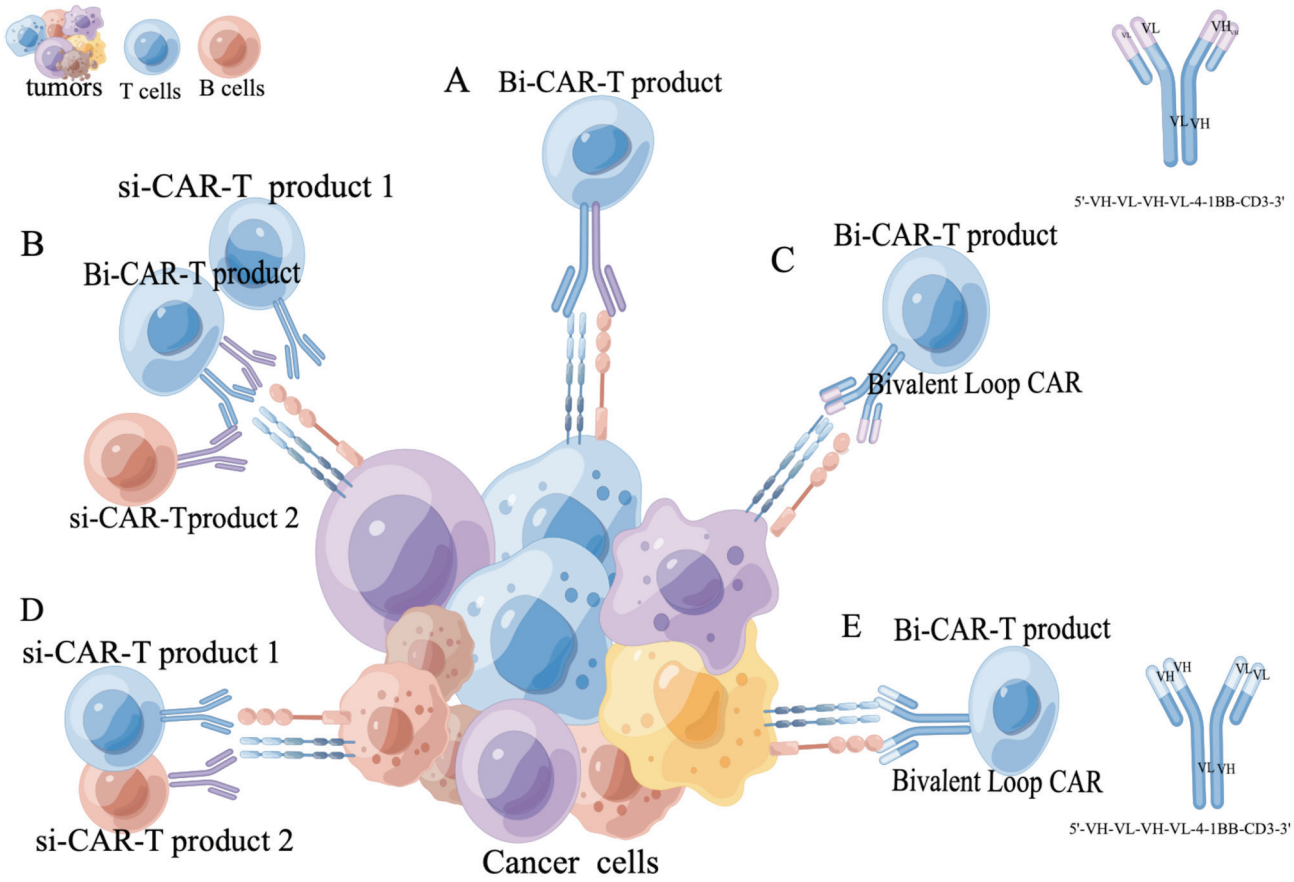

Figure 3. CAR-T cell therapy based on dual targeting. (A) The sequential infusion of two Si-CAR T-cell products, each transduced with a different vector. (B) Co-transduction of two vectors encoding one CAR each produces a pool of two Si-CAR T-cell products and one Bi-CAR-T cell product. (C) Bi-CAR-T product containing two separate CARs, each with an antigen-binding domain, produced by transduction of the bicistronic vector. (D) A bivalent tandem CAR, where the VL-VH of one scFv linked to the VL-VH of the other scFv. (E) A bivalent loop CAR consisting of one scFv's VL-VH separated by another scFv's VL-VH. Bi-CAR-T, bispecific chimeric antigen receptor T-cell; scFv, single-chain variable fragments; Si-CAR-T, single-targeted CAR T-cell; VH, variable heavy chain; VL, variable light chain.

Chimeric antigen receptor (CAR)-T and TCR-T therapy. CAR-T cells targeting CD19 have been successful in treating recurrent hematological malignancies, with an objective response rate of >70%. Promising conversion rates have also been achieved with B-cell maturation antigen's chlorpyrifos and sitagliptin's self-microspheres, reaching 73 and 97%, respectively (57). CAR-T cell therapy was shown to have limitations, such as recurrence due to limited persistence or functional inhibition of CAR-T cells or antigen escape (58). The use of dual-target CAR-T cell therapy was shown to be more effective than single-target CAR-T cells, and to also have a lower incidence of severe cytokine release syndrome and no neurotoxicity (59). However, to achieve optimal results in dual target CAR-T cell therapy, further optimization of CAR target selection and CAR structure is necessary (60). Multi-target CAR-T cell therapy is a crucial area of research with the potential to revolutionize tumor immunotherapy in the future (Fig. 3).

Signaling pathway-focused therapy. Signaling cascades are crucial for various cellular processes, such as proliferation, differentiation, migration, intercellular communication and survival. Tumor heterogeneity in GBM can lead to various outcomes, such as increased proliferation, abnormal angiogenesis and evasion of cell apoptosis pathways. The RTK (RAS-PI3K mTOR) pathway is of particular interest in GBM research due to mutations in EGFR, VEGFR and PDGFR. Small-molecule tyrosine kinase (TK) inhibitors, such as Nivozumab and Rigofenib, selectively block single or multiple TK receptors (61). In addition, PI3K inhibitors, such as Bupanib and GDC-0084, dual PI3K/mTOR inhibitors such as Dacoxib, and mTOR inhibitors such as AZD8055, have been identified (62). As Myc is highly expressed in glioblastoma, the combination of the CDK9 inhibitor zoteracib and temozolomide have been shown to reduce the level of Myc (63,64). Cell surface antigens, such as TK receptors found on glioma cells, have the potential to be therapeutic targets for antibody-drug conjugates. Clinical trials involving L19TNF have demonstrated low toxicity and the combination of L19TNF with other chemotherapy drugs, such as ICIs, may improve efficacy (65).

Epigenetic therapy. The search for new interventions at the genomic level has resulted in the development of epigenetic treatments. These treatments involve modifications of gene expression, transcription and translation, which have the potential to suppress oncogenes and support suppressor genes. By preventing proliferation, causing cell cycle arrest and promoting apoptosis, these interventions can effectively

combat cancer. In addition, miRNAs with epigenetic effects and histone deacetylase inhibitors are potential drug candidates that can prevent glioma cell proliferation. The drugs Vorinostat and Panobinostat were investigated in conjunction with another medication for the treatment of glioblastoma (66).

Radiosensitizers. Glioblastoma is known for its anti-radiation properties, which are attributed to various mechanisms, such as microRNA, tumor heterogeneity, glioma stem cells, tumor microenvironment, hypoxia, metabolic changes and DNA damage/repair. One crucial player in DNA repair and prevention of cell apoptosis is poly(ADP ribose) polymerase PARP. The PARP inhibitor Olaparib has shown promising results in the treatment of breast cancer. Research is currently being conducted on the use of Veliparib and Fluzopanib in combination for the treatment of GBM (67). The detection and repair of DNA double-strand breaks caused by radiation-induced DNA damage is crucial and can be facilitated by DNA-dependent protein kinases (DNA-PK). Inhibitors of DNA-PK, such as AZD7648 and NU7441, have been studied (68). ATM and ATR kinases have a crucial role in regulating DNA damage repair and maintaining genomic stability. A promising development in this field is the testing of AZD1390, an ATM molecular inhibitor, in phase I trials for patients with GBM (69).

Novel radiation therapy. The optimization of GBM radiation therapy has a crucial role in reducing side effects on normal brain tissue. Focal brain radiation therapy has been implemented due to progress in accuracy. Three-dimensional techniques such as intensity-modulated radiation therapy and volume arc therapy have led to a decrease in non-tumor tissue dose (70). The particle therapy approach, including techniques such as gamma knife and Zap-X, aims to minimize the dose to the edge of the tumor. This can help to reduce the amount of radiation received by adjacent tissues and organs at risk of the tumor. Proton beam therapy and carbon ion radiotherapy are currently being extensively researched for their potential to achieve this goal (71).

5. Conclusions and prospects

Tumor development is influenced by both genetic and non-genetic factors, along with an imbalanced immune metabolism. It is essential to identify potential targets for new therapeutic regimens to shape the metabolic characteristics and immune heterogeneity of the tumor microenvironment. By utilizing liquid biopsy techniques to detect heterogeneous vesicles, valuable insight into the presence of immune components in the tumor microenvironment may be obtained. The present review proposes the combination of radiotherapy and chemotherapy, as well as CAR-T single-target and multi-target therapy, to address the heterogeneity of tumor patients. Furthermore, liquid biopsy technology is suggested as a means to provide an accurate treatment basis and propose personalized treatment plans. Further research is needed to determine the applicability of these strategies in patients with different types of solid cancers and develop effective treatment approaches. Precise and personalized treatment plans are crucial, as they not only improve the prognosis and maximize the treatment effect for tumor patients but also enhance overall survival time and quality of life.

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Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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