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Review

Tumor antigens in glioma

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ABSTRACT

Immunotherapy applications to glioblastoma represent a new treatment frontier. Antigen-targeted immunotherapy approaches hold enormous potential to elicit antigen-specific anti-tumor effects in central nervous system tumors. Still, the paucity of effective antigen targets remains a significant obstacle in safely and effectively treating glioblastoma and other malignant gliomas with relatively low mutation loads. In this review, we highlight the current understanding of and development of immunotherapy to target 1) shared non-mutant antigens 2) shared mutant antigens (neoantigens) derived from cancer-specific mutations 3) personalized neoantigens derived from tumor-specific genetic alterations containing *de novo* peptide sequences and 4) virus-derived antigens. We also discuss strategies to enhance tumor immunogenicity and neoantigen prediction. Spatial heterogeneity remains a formidable challenge for immunotherapy of glioma; recent advances in targeting multiple antigens and refining the antigen selection pipeline hold great promise to turn the tide against glioma.

1. Introduction

Diffuse gliomas are the most common primary malignant central nervous system (CNS) tumors, accounting for 80 % of all malignancies in the CNS [1]. They are classified according to their histology and molecular characteristics as grade II–IV by the World Health Organization (WHO) [2,3]. Grade IV glioblastomas (GBMs) are the most aggressive, with a median overall survival (OS) of fewer than two years, even with the current standard of care (radiation therapy [RT] with temozolomide [TMZ] chemotherapy and an antimitotic treatment device, Tumor Treating Fields [TTF] therapy) [4–6]. Grade II–III gliomas (*i.e.*, astrocytomas and oligodendrogliomas) also exhibit malignant behavior: they grow invasively, progress to higher grades, and most patients eventually succumb to the disease [7].

Brain tumors can arise in all age groups and they are especially devastating in children, for whom they are the leading cause of cancer-related mortality and morbidity [8]. The prognosis for children with diffuse midline gliomas (DMG), including diffuse intrinsic pontine gliomas (DIPG), is especially poor – the median OS for DIPG is less than one year [9,10]. Although much effort has been expended in optimizing treatment regimens, no real progress has been made and the clinical prognosis remains extremely poor in glioma patients [11]. Development of novel and effective treatment modalities is urgently warranted.

Immunotherapy holds promise as a treatment for gliomas. Although the CNS has been recognized as an “immune privileged” site, recent findings suggest that the “privileged” status is not absolute [12–14]. It has been demonstrated that effector immune cells, such as T-cells, can penetrate the blood-brain-barrier (BBB) and mediate antigen-specific immune responses [15]. Furthermore, cumulative findings from preclinical and early-phase clinical studies of antigen-targeted immunotherapies have indicated that these approaches can elicit antigen-specific anti-tumor effects in CNS tumors [16–23].

Thus far however, no randomized prospective clinical trials have succeeded in demonstrating a robust clinical benefit. Recently, two phase III trials of glioma immunotherapy (ACT-IV trial of rindopepimut, an EGFRvIII vaccine [24] and CHECKMATE-143 trial of nivolumab, an anti-PD-1 monoclonal antibody therapy for recurrent GBM [25]) failed to show objective benefits. The failures of these studies further underlined the challenges faced by those developing immunotherapy for this disease. These challenges include, but are not limited to, the following three major categories. The first is the paucity of tumor-specific antigens. This problem is further complicated by the marked spatial, temporal, and inter-tumor heterogeneity of malignant gliomas [26–30],

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which necessitates multiple antigen-targeting approaches. The second is glioma-induced immunosuppression. This includes the expression of inhibitory checkpoint molecules [31–36] and the secretion of immunosuppressive cytokines in the tumor microenvironment [37–40] as well as the enrichment of immunosuppressive myeloid cells and regulatory T-cells at the tumor site [41,42]. The third is the unique immunological environment of the CNS and the homing of therapeutic immune effector cells to the tumor. This encompasses complexed functions of the blood-brain barrier [12–14] and the absence of lymphatic organs and effective antigen-presentation systems for T-cell responses in the CNS [15,43–45]. While all of these issues must be addressed in order to develop effective immunotherapy for gliomas, in this article, we focus on the unique features of glioma antigens, reviewing the latest advances and revisiting the importance of the following components: shared non-mutant glioma antigens, neoantigens, which are derived from cancer-specific somatic mutations, and viral antigens. We also outline a few of our own propositions on how to refine targeted immunotherapeutic strategies for the treatment of gliomas.

2. Current understanding of and therapeutic development targeting tumor antigens in glioma

One of the most critical issues for developing safe and effective cancer immunotherapy is ensuring tumor-specific cytotoxicity. In some clinical trials of T-cell therapy targeting non-mutant cancer-associated antigens, on-target off-tumor cross-reactivity of T-cells administered as part of the treatment caused life-threatening events [46,47], including two cases of lethal brain edema [47]. These incidents underlined the importance of carefully establishing and expanding the list of glioma-specific antigens.

Cancer antigens can be divided into three classes: non-mutant proteins (to which T-cell tolerance is incomplete owing to their absence or suppressed expression in normal tissues), neoantigens (mutant proteins that are entirely absent from normal human tissues) and virus-derived antigens. In this section, we primarily discuss our current understanding of and progress on 1) non-mutant, shared glioma-associated antigens; 2) shared neoantigens such as IDH1 R132H, H3.3 K27 M, and EGFRvIII; 3) non-shared, patient-specific neoantigens; and 4) cytomegalovirus (CMV)-derived antigens (Table 1). Some of these are cell surface antigens that can be targeted by CAR-T-cell therapies, and others are derived from intracellular proteins that can be targeted by vaccines and/or T-cell receptor-transduced T-cell (TCR-T) therapy. As an introduction, we will begin with the results and implications of a recent clinical trial that incorporated many of the topics we hope to cover in this section.

2.1. GAPVAC-101 trial

In 2019, Hilf, Wick, and Okada et al. reported the results of the GAPVAC-101 phase I clinical trial, in which 15 newly diagnosed GBM patients with HLA-A*02:01 or 24:02 were treated with “personalized” vaccine cocktails consisting of mutant and non-mutant peptides [48]. This study is of particular interest and importance to this review because of its two separate steps of antigen selection, one for actively personalized vaccine 1 (APVAC1) and another for APVAC2. For APVAC1, Immatics, Inc. established a warehouse of premanufactured, synthetic peptides composed of non-mutant antigens. The antigens had been confirmed to fulfill the following criteria: 1) their epitopes were presented in the HLA class I peptidome in GBM; 2) they had high expression in GBM; 3) they had very low or absent expression in healthy tissues; 4) they were thought to play critical biological roles in gliomagenesis; and 5) they had high immunogenicity (they could efficiently induce and stimulate antigen-specific T-cell responses in *in vitro* assays) [49]. As part of APVAC1, each patient received 6–7 individually selected best-ranking HLA class I peptides from the warehouse. Granulocyte–macrophage colony-stimulating factor (GM-CSF) and poly-ICLC were co-administered to enhance immune responses synergistically [50]. As part of APVAC2, each patient received two additional mutant or non-mutant peptides. The mutant-antigens were preferentially selected based on 1) individual HLA peptidome analysis by mass spectrometry (MS) or 2) predicted HLA class I binding and immunogenicity. If no suitable neoepitopes were found, non-mutant antigens identified in the individual immunopeptidome (but not chosen for APVAC1) were employed.

Unfortunately, the study team was unable to detect any neoantigen presentation on patient HLA class I or II. In most of the patients, however, an increased number of CD8 + T-cells reactive to at least one APVAC1 non-mutant peptide was observed, accompanied by a shift to a memory phenotype. APVAC2 mutant peptide vaccines, meanwhile, were found to preferentially induce CD4+ T-cell responses.

The results of the GAPVAC-101 trial suggest that it may be possible to combine both non-mutant antigen and neoantigen targets to elicit potent immune responses against heterogeneous glioma antigens. The “off-the-shelf” non-mutant peptide warehouse system was particularly useful as it allowed multiple personalized selections of antigen peptides that had been validated for their immunogenicity and HLA presentation [48,51]. Continuing advances in bioinformatics offer much hope that the identification and validation of somatic mutation-derived neoantigens will only improve in the coming years. As such, we argue that it is crucial to understand components of non-mutant as well as mutant antigens in gliomas, and to incorporate both in the development of future treatments.

Table 1
Classification of tumor antigens in glioma.

	non-mutant	mutant, shared	mutant, non-shared (private)	virus-derived
antigens	IL13Rα2, HER2, EphA2, survivin, TRP2, WT1, gp100, SOX2, SOX11, MAGE-A1, AIM2, *etc*.	IDH1 R132H H3.3 K27M EGFRvIII ones derived from SNVs in *EGFR* and *TP53* genes [61,62]	private neoantigens	viral antigen pp65, IE1, or glycoprotein B, from CMV
frequency	highly shared (see Table 2)	IDH1 R132H – 70 % of WHO grade II–III glioma H3.3 K27 M – 70–90 % of DIPG EGFRvIII – 20 % of IDH-wildtype GBM	private/unique to individual patients	varied among studies (> 90 % of GBM)
specificity	quantitatively overexpressed in tumors but lower levels of expression may be seen in normal cells	absolute (unless TCR has cross-reactivity)	absolute (unless TCR has cross-reactivity)	not detected in normal brain tissue
availability of “off-the-shelf” approach	available	available	not available	available

Abbreviations: SNVs, single nucleotide variants; CMV, cytomegalovirus; WHO, world health organization; DIPG, diffuse intrinsic pontine glioma; GBM, glioblastoma; TCR, T-cell receptor.

2.2. Shared non-mutant antigens

In addition to the antigens listed in the GAPVAC warehouse [48], there are a number of non-mutant antigens that have been evaluated as targets for vaccine or adoptive T-cell therapy approaches (Table 2). EphA2, for instance, has been observed to be generally negative in normal glial cells and overexpressed in approximately 90 % of GBM samples [52], making it a potentially attractive target for immunotherapy. Many non-mutant antigens found in GBM are also overexpressed in other cancers. For instance, HER2 is overexpressed in approximately 30 % of breast cancer patients as well as in several other malignancies [53]. However, it is also expressed at low levels in several normal tissues including the gastro-intestinal and respiratory tracts [54], raising the risk of on-target off-tumor toxicity. Indeed, in a clinical study, a patient with colorectal cancer died due to cardiopulmonary complications after the administration of third-generation HER2-CAR T-cells with a trastuzumab-based antigen recognition exodomain and a CD28.41BB.ζ signaling endodomain. The fatal complications presumably arose because the CAR T-cells recognized low levels of HER2 on the patient's lung epithelial cells and ended up triggering cytokine release syndrome (CRS) [46]. Following this incident, the HER2-specific CAR was optimized to avoid such serious adverse events, employing a second-generation HER2-CAR with an FRP5-based exodomain and a CD28.ζ endodomain. This second-generation HER2-CAR demonstrated anti-tumor activity in sarcoma while crucially avoiding any toxic side effects [55]. Treatments like the HER2-CAR, which target GBM-associated antigens and have been evaluated in other types of cancers, lay the foundation for future cross-cancer antigen studies.

Immunotherapy targeting just one antigen in solid cancer is insufficient due to the marked antigenic heterogeneity of tumors. Hegde et al. reported that CAR T-cells targeting HER2 and IL-13Rα2 simultaneously (also known as tandem CAR) exhibited offset tumor antigen escape and enhanced functionality in a preclinical orthotopic GBM xenograft mouse model [56]. The same group also analyzed the surface expression of HER2, IL13-Rα2, and EphA2 in 15 primary GBM samples and showed that their novel treatment strategy co-targeting these three antigens was capable of capturing and eradicating nearly 100 % of tumor cells [57].

Vaccine approaches may have a unique advantage over genetically engineered T-cell approaches in terms of their ability to target more extensive numbers of antigens simultaneously. The IMA-950 multipeptide vaccine, for example, includes 11 peptide targets [49], including 9 MHC class I and 2 MHC class II peptides identified on primary GBM tissue. In a phase I clinical study of IMA950 combined with GM-CSF in HLA-A*02-positive patients with newly diagnosed GBM who received chemoradiotherapy, 90 % of the patients showed antigen-specific CD8 + T-cell responses against at least one antigen, and over 50 % of the patients showed specific CD8 + T-cell responses against more than one antigen [23]. In a follow-up phase I/II clinical trial using IMA950 with poly-ICLC, single- and multiple-antigen-specific CD8 + T-cell responses were observed in 63.2 % and 38.2 % of patients respectively. Single- and multiple-antigen-specific CD4 + T-cell responses were observed in 57.9 % and 36.8 % respectively [58].

Shared non-mutant antigens can thus be attractive targets for immunotherapy. Antigen selection in both the GAPVAC-101 and IMA-950 trials was based on careful screening and validation, including HLA peptidome analyses and antigen-specific T-cell response assays [59].

2.3. Shared mutant antigens (neoantigens)

Antigens derived from cancer-specific somatic mutations are in many ways ideal targets for immunotherapy. Their expression is almost entirely limited to tumor cells, greatly decreasing the risk of on-target off-tumor toxicity. Being spontaneous mutations, however, they are often minimally shared among the patient population, rendering most unsuitable as targets for immunotherapy. To date, *IDH1* R132H, *H3F3A* (H3.3) K27 M, and *EGFR*vIII are the only mutations that have been found to be frequently shared among specific patient subgroups. Although several other mutations, including *TP53* R273C and *EGFR* A289 V, are also recognized as recurrent and may be targeted in the future [60], these have been less thoroughly investigated as targets for glioma immunotherapy [61,62]. In this section, therefore, we limit our discussion to the first three mutations and their potential as "shared neoantigen" targets in glioma.

2.3.1. IDH1 R132H

The gain-of-function monoallelic R132H point mutation in the isocitrate dehydrogenase 1 (*IDH1*) gene (as well as its correlation with a relatively favorable prognosis) was first reported in GBM by Parsons et al. in 2008 [63]. Along with a rare mutation in the isocitrate dehydrogenase 2 (*IDH2*) gene, it turned out to be a hallmark of grade II-III gliomas (present in 70 % of cases) and, albeit to a lesser extent, of secondary GBM (present in 10 % of cases) [64–66]. The *IDH1* R132H mutation was found to be the most common among glioma patients, accounting for more than 90 % of all *IDH1* mutations observed [64,67]. Mutant IDH1/2 enzymes convert α-ketoglutarate (αKG) into the oncometabolite $_{D}$-2-hydroxyglutarate (2-HG). 2-HG competitively inhibits the enzymatic activity of many αKG-dependent dioxygenases and leads to histone dysregulation, genome-wide DNA hypermethylation, and aberrant angiogenesis [65,68,69]. Subsequent studies have revealed that *IDH1/2*-mutant and -wildtype gliomas have distinct biological behavior [70]. Hence, IDH status was included in the recent revision of WHO classification as an essential diagnostic marker along with several other molecular markers such as chromosome 1p/19q status and histone H3 status [2,71].

The immunosuppressive effects of the *IDH1/2* mutation have also

Table 2
Expression and clinical application of representative non-mutant antigens in glioma.

antigen	expression in GBM	expression in other types of cancers	clinical study status for GBM
IL13Rα2	50-80 % [161,162,163]	colon, pancreas, ovary, melanoma [164]	CAR-T phase I [16,165] median OS after relapse ~11 months
HER2	30-40 % [53]	bladder, breast, cervical, colon, esophagus, gastric, renal, lung [166]	CAR-T phase I/II [167] median OS after relapse ~11 months
EphA2	90 % [52]	ovary, prostate, breast, pancreas, lung, bladder, melanoma, esophagus, colon, kidney, thyroid, vulvar, cervix, oral [52]	CAR-T phase I/II (NCT0257526)
survivin	85 % [168]	melanoma, breast, esophagus, pancreas, liver, lung [169]	vaccination phase I/II/III [170] median OS after relapse ~21months
WT1	94% [171]	leukemia, solid tumors (breast, ovarian, soft tissue sarcoma) [172]	vaccination phase I/II [173] median OS after relapse ~7months

Abbreviations: CAR, chimeric antigen receptor; OS, overall survival.

been recognized [34,72,73]. Our group has previously reported that 2-HG produced by IDH-mutant glioma cells inhibits STAT1 and downstream leukocyte-recruiting chemokines, such as CXCL10 [34]. Interestingly, administration of mutant IDH inhibitor not only restored T-cell accumulation (typically reduced in IDH-mutant tumors compared with wildtype ones) but also enhanced the efficacy of peptide vaccines in an *in vivo* model. Bunse et al. subsequently reported that T-cells actively ingest exogeneous 2-HG using sodium-dependent dicarboxylate transporter 3 (SLC13A3), resulting in reduced proliferation and cytokine secretion. They also showed that inhibition of 2-HG restored T cell activity and enhanced anti-tumor immunity induced by mutant IDH1-specific vaccine [57].

Despite such immunosuppressive features, the *IDH1* R132H mutation attracts much attention as a target for immunotherapy because of its high incidence and high cellular clonality [64,74]. Of particular importance is that the mutation is truncal, with all tumor cells expressing the mutant IDH1 peptide [67] (with the exception of rare cases where IDH-mutations are lost [75]). Schumacher et al. reported that vaccinations using a peptide encompassing the *IDH1* R132H mutation (p123-142) could induce both epitope-specific CD4+ T-helper-cell responses (T_H1) as well as antibody responses, thereby eradicating the mutation-positive tumors *in vivo* in an human leukocyte antigen (HLA)-A2.DR1-transgenic mouse model [19]. Subsequently the first-in-human phase-I trial NOA-16 was initiated to test the mutant-IDH1 R132H peptide vaccine in patients with newly diagnosed, *IDH1* R132H mutant WHO grade III–IV astrocytomas (NCT02454634). It has thus far shown optimal results as far as its safety and immunogenicity, including the induction of antigen-specific TCRs [76].

Notably, hopes for T-cell therapies targeting this intracellular mutant peptide remain unrealized. Schumacher et al. described that they did not observe any epitope-specific CD8 + T-cell induction [19]. However, should the TCR that specifically recognizes the epitope presented by HLA class I or II be induced and isolated, it should be possible to develop IDH1 R132H-TCR-T-cell therapy. This would bring great advantages for many patients, considering the extremely high penetrance of the mutation in this disease. Though vaccination seems promising, further studies employing novel discovery approaches are warranted to discover such beneficial TCRs for more proactive immunotherapy.

2.3.2. H3.3 K27 M

Gliomas in children have different molecular drivers from those arising in adults. DMGs, which affect primarily children and adolescents, arise in the midline structure of the CNS, including the brain stem, thalamus, and spinal cord [1,77]. They are characterized by a highly recurrent point mutation in the *H3F3A* gene encoding histone H3.3 (and less frequently, a mutation in the *HIST1H3B* or *HIST1H3C* genes coding H3.1). An amino acid substitution from lysine to methionine at position 27 (K27 M) leads to a decrease in H3 K27 trimethylation, resulting in distinct global demethylation and an aberrant gene expression pattern due to inhibition of polycomb repressor complex 2 (PRC2) activity [78–82]. The mutation is observed in 70–90 % of diffuse intrinsic pontine gliomas (DIPGs), which are the most common form of DMG and account for 10–20 % of all pediatric brain tumors [78,80,81].

As TMZ and other chemotherapies have proven ineffective for H3 K27 M mutant DMG, the current standard therapy for this disease is local irradiation alone [83,84]. Compared to H3.3 wildtype or H3.1 mutant DIPGs, H3.3 mutant tumors are less sensitive to radiotherapy and exhibit more aggressive behavior [78,81]. As a consequence, the prognosis of patients with H3.3 K27M-mutant DIPG is devastating: the median OS is less than one year and the 2-year survival rate is less than 10 %, representing a terribly unmet clinical need.

Encouragingly, the mutation is also an attractive target for immunotherapy. Similarly to the *IDH1* R132H mutation in adult gliomas, the H3.3 K27 M mutation is homogeneously distributed throughout the entire tumor [77,85]. Our group and several other researchers have investigated the H3.3 K27 M epitope. Recently, Ochs et al. reported that H3.3 10mer (H3.3 K27 M p26–35) and 27mer (H3.3 K27 M p14–40) peptide vaccines are capable of binding to HLA-A*0201 and both HLA-A*0201 and HLA-DR1, respectively, and eliciting mutation-specific cytotoxic T-cell- and T-helper-1-cell-mediated immune responses in preclinical models [86]. Our group concurrently identified the H3.3 K27 M p26–35 epitope, and isolated cDNA for α- and β-chains of a high affinity TCR that recognizes the H3.3 K27 M mutation in the context of HLA-A*02:01 [17]. In this study, we stimulated HLA-A*02:01 + CD8 + T-cells with the synthetic H3.3 K27 M mutant 10mer peptide (H3.3 K27 M p26–35), isolated a CTL clone with a high H3.3 K27M-tetramer-binding activity, and cloned the TCR cDNA into a retroviral vector. TCR-transduced T-cells efficiently killed HLA-A*02:01 + H3.3 K27M + glioma cells in an antigen- and HLA-specific manner both *in vitro* and *in vivo*. These data provide a solid basis for the development of vaccines as well as TCR-T-cell therapies targeting the H3.3 K27 M mutation.

The Pacific Pediatric Neuro-Oncology Consortium (PNOC) has since started a pilot clinical trial to assess the safety and the efficacy of an H3.3 K27M-peptide vaccine in newly diagnosed HLA-A*0201+ patients with H3.3 K27M + DIPG (PNOC007 trial, NCT02960230) [87]. In this study, the enrolled patients receive the vaccine and concurrent poly-ICLC following treatment with the standard of care (radiation therapy). This trial also aims to characterize the vaccine-induced H3.3 K27M-specific T-cells in peripheral blood at a multitude of time points utilizing a novel H3.3 K27M-specific dextramer. Further development of this methodology will enhance investigation of immunotherapeutic outcomes on molecular and cellular levels.

2.3.3. EGFRvIII

Epidermal growth factor variant III (*EGFR*vIII) is the most common *EGFR* mutation, observed in approximately 20 % of newly diagnosed IDH-wildtype GBMs [88,89]. The mutant protein arises from an in-frame deletion of exons 2–7 of the *EGFR* gene and reside primarily on abundant small circular extrachromosomal DNA fragments (double minute chromosomes) [90,91]. This genomic rearrangement results in overexpression of a truncated cell surface protein missing major parts of its extracellular domain. The protein constitutively activates a tyrosine kinase and signaling through the RTK/RAS/PI3K pathway, leading to tumorigenesis [92–94]. Because this mutation is tumor-specific, never present in normal cells [89,95], and because the EGFRvIII protein harbors a *de novo* peptide sequence generated by genomic rearrangement, it has been regarded as a good target for immunotherapy, including vaccination, antibody-based, and T-cell-based therapies [96].

Rindopepimut is a 14 amino acid peptide vaccine derived from EGFRvIII that encompasses the mutation site and is conjugated to keyhole limpet hemocyanin (KLH) [97]. It has achieved a prolonged OS in comparison with the historical control in a phase II study [98]. However, an international phase III trial (ACT IV) in 745 patients with newly diagnosed GBM failed to demonstrate survival benefit over the control group [24].

Several mouse monoclonal antibodies have been developed that specifically recognize an extracellular epitope of EGFRvIII [99–101]. Using scFvs from the antibodies which demonstrated tumor specificity and anti-tumor efficacy in both *in vitro* and *in vivo* xenograft models, CAR constructs targeting the *EGFR*vIII mutation have been established [96,102–104]. Our collaborative group between U. Penn and UCSF reported a pilot trial in which 10 patients with recurrent GBM were treated with EGFRvIII-targeting CAR-T-cell therapy (NCT02209376)

[18]. After a single intravenous infusion of $1.75-5 \times 10^8$ CAR T-cells, no patients experienced off-tumor toxicity or systemic CRS. Although no obvious effect on the tumor growth was observed in follow-up MRI scans, one patient maintained stable disease for 18 months after CAR-T-cell administration without any additional treatment. Furthermore, in five of seven patients who undertook surgery after the infusion, tumor-infiltrating CAR-T-cells, as well as abundant non-transduced T-cells, were observed in the resected tumor samples. In addition to the CAR-T-cell infiltration, EGFRvIII expression was attenuated compared with the corresponding pre-treatment tumor samples, suggesting that immunoediting was another consequence of the treatment. Phase I clinical trials of EGFRvIII-targeted CAR-T-cell therapy are currently in progress for newly-diagnosed (NCT02664363) and recurrent (NCT 01454596, NCT02209376, NCT02331693, NCT02844062, NCT03283631) GBM.

2.4. Personalized neoantigens

Neoantigens are defined as antigens derived from tumor-specific genetic alterations, and thus contain *de novo* peptide sequences that are absent in the human normal cells [105]. They are regarded as ideal targets for immunotherapy since the hosts' immune system does not show central T-cell tolerance to these antigens and can recognize them as foreign. Indeed, it has been shown that the mutation load, which should correlate with the number of neoantigens, is a predictive factor for immune checkpoint inhibitors (ICI) in the majority of cancer types. [106–108]. Unfortunately, GBMs are considered to harbor a low mutational burden: they contain only 30–50 non-synonymous mutations [109]. In addition, as already described, the number of recurrent/shared mutations is very limited in gliomas with the exemption of *IDH1* R132H, *H3F3A* K27 M, and *EGFR*vIII. Accordingly, non-shared "private" mutation-derived neoantigens have been evaluated as therapeutic targets in recent studies.

A major obstacle to the development of personalized neoantigen immunotherapy is the unreliable prediction of "true" neoantigens by currently available workflows. As discussed earlier, researchers struggled with neoantigen validation while designing APVAC2 for the GAPVAC-101 trial, failing to demonstrate the presence of predicted neoantigen epitopes in the HLA peptidome [48]. In gastrointestinal cancers, only 1–2 % of mutations have been proven to elicit immunological reactions in the corresponding tumor-infiltrating lymphocytes (TILs) [110].

Nevertheless, Keskin et al. recently reported encouraging results from their neoantigen-based vaccine clinical trial in their article published back-to-back with the GAPVAC-101 trial [111]. They assessed the feasibility of personalized neoantigen-targeting vaccines in patients with newly diagnosed GBM. In their analytic workflow, a median of 64.5 (range, 30–163) mutant epitopes were predicted to be HLA class I high-binders ($IC_{50} < 500$ nM), although they did not perform MS-based validation for HLA-bound peptides. Two patients who did not require corticosteroids during the vaccine priming period exhibited robust *de novo* T-cell responses against multiple predicted neoantigens, in which both CD8+ and CD4+ T-cells were induced and enriched in an antigen-experienced memory phenotype with polyfunctionality. In addition, post-vaccination tumor specimens from these two patients showed significant increases in tumor-infiltrating T-cells, whereas no increase was observed in patients who received corticosteroid administration. TCR repertoire analyses suggested successful trafficking of vaccine-induced neoantigen-reactive T-cells to the tumor site.

These studies suggest that GBMs, immunologically "cold" tumors, can be successfully infiltrated by neoepitope-specific T-cells after personalized vaccine treatment. Development of more comprehensive strategies, such as combination with ICI, is needed to control inhibitory factors and maintain the reactivity of infiltrating T-cells.

2.5. Viral antigen epitopes

Viral antigens may represent particularly attractive targets for immunotherapy because they are foreign to the host immune system and thus are inherently immunogenic [45]. Though gliomas have not been recognized as being virally induced, a growing body of evidence suggests that sensitive assays detect expression of CMV genomic and protein materials in >90 % of GBMs. Importantly, expression is not detected in normal brain tissue [112–115]. A recent study of a DC vaccine targeting CMV epitopes in GBMs demonstrated promising results, especially in combination with vaccine-site conditioning using tetanus-toxioid [116]. These findings are now being further investigated in phase I and II clinical trials (NCT00639639, NCT02465268, NCT02366728, and NCT03382977) [117,118].

3. Ongoing work and future directions of antigen-oriented immunotherapy for glioma

3.1. Enhancement of tumor immunogenicity

Additional challenges to the success of cancer immunotherapy for glioma patients include the immunosuppressive tumor microenvironment, the relatively low mutation load of gliomas, and the poor immunogenicity of some neoantigens (relative to viral antigens) [119]. It is therefore an important goal to develop and integrate strategies that enhance tumor immunogenicity.

The relationship between irradiation and immunotherapy has long been an interesting area of research. Irradiation induces damages in DNA as well as the cell membrane, producing reactive oxygen species (ROS) [120]. By activating multiple transcription factors and signal pathways, it can change the immunogenicity of the tumor as well as the immunophenotype of cell components in the tumor microenvironment. Major immunological consequences of irradiation may include 1) enhancing antigen presentation due to upregulation of MHC class I molecules, 2) phagocytosis and immunity induction by calreticulin expression and high mobility group box 1 (HMGB1) protein release, and 3) induction of apoptosis caused by upregulated Fas ligand expression [121]. In addition, the breakdown of tumor cells by irradiation can lead to the release of tumor antigens and damage-related molecules (DAMPs) such as HMGB1 [122]. It is expected that these molecules can stimulate dendric cells and activate cellular immune responses *via* Toll-like receptors, resulting in enhancement of tumor antigen presentation.

3.2. Treatment induced-hypermutator as an optimal subject for immunotherapy

Compared with some other types of cancers with a high mutational burden such as melanoma, smoking-related non-small cell lung cancer, and microsatellite instability (MSI)-high colon cancer [123], GBMs have fewer non-synonymous mutations (median mutational burden 2.7 mut/Mb) and thus have limited potential to generate neoantigens [53,124]. Some exceptional GBM cases, however, harbor a significantly higher number of mutations (≥ 10 mut/Mb) and are known as hypermutators [27,125–127]. Hypermutated GBMs can arise due to TMZ-induced disruption of [27,128] or hereditary deficiencies in [127,129] the DNA mismatch repair (MMR) pathway. TMZ is an alkylating agent used for GBM patients as a major component of the current standard of care [27,28,127,128], and its disruption of the MMR system is presumably the most common cause of hypermutation in GBM [130]. It is challenging to accurately estimate the incidence of TMZ-induced hypermutated GBM, but it is thought to be responsible for 10–20 % of GBMs that recur after TMZ treatment [15,28,131]. Hereditary MMR deficiency is the less common of the two. More often seen in pediatric

cases, these tumors tend to be ultra-hypermutated (≥ 100 mut/Mb) [127].

Two case studies have suggested that such tumors may be good targets for ICI therapy [132,133]. In 2016, Bouffet et al. reported two siblings with hypermutated GBM arising from germline biallelic MMR deficiency for which ICI therapy with nivolumab showed durable clinical responses [132]. That same year, Johanns et al. described a patient with hypermutated GBM from a germline *POLE* gene mutation for whom treatment with pembrolizumab (an anti-PD-1 inhibitor) resulted in a significant increase in tumor-infiltrating CD8+ cytolytic T-cells as well as elevated expression of PD-1, PD-L1, and IFNγ in resected tumor tissue [133]. In 2017, pembrolizumab was approved for the treatment of adult and pediatric patients with unresectable or metastatic, MSI-high or MMR-deficient solid tumors, regardless of tumor site or histology [134,135]. As a result, hypermutated GBM with MMR deficiency can currently be treated with ICI therapy as a first-line treatment.

There are currently no data on whether TMZ-induced hypermutated GBM can be a good target for ICI therapy. However, Wang et al. reported that such cases were shown to be enriched in CD8 + T-cell infiltration (based on *in silico* deconvolution prediction) [136]. They suggested that this subset of patients may be responsive to ICI because of their more immunologically reactive microenvironment. At the same time, it is also possible that these patients may suffer from TMZ-induced lymphopenia, which would exacerbate local immunosuppression. Two ICIs, pembrolizumab and avelumab (an anti-PD-L1 inhibitor), are currently being evaluated, either alone in patients with recurrent WHO grade II–IV hypermutated glioma (NCT02658279), or in combination with radiotherapy in patients with recurrent, secondary GBM (NCT02968940) [137]. The results of these studies will prove highly consequential for the future of hypermutator-inducing therapies, and are eagerly awaited.

Cancer vaccine development in cases of hypermutated GBM may also be a favorable option as there would be a significantly expanded pool of candidate peptides for the vaccine. Additionally, the finding that tumor-reactive T-cells are recruited to the hypermutated tumor suggests that synergistic effects could be expected with ICI and vaccination therapy.

3.3. "Next-generation" neoantigen prediction

Along with increased access to high-throughput next-generation sequencing (NGS) techniques over the last decade, cancer immunogenomics has greatly contributed to the development of neoantigen-directed immunotherapy [138–140]. Variant calling, quantification of mRNA expression, HLA-typing, and the estimation of the HLA binding affinity of *de novo* peptides can be usually carried out through whole exome-sequencing (WES) of the tumor and of normal genomic DNA (*e.g.*, from PBMC) and RNA-sequencing (RNA-seq) of the tumor [15,141,142]. For HLA class I binding, putative tumor-specific 8–11mer neoepitopes derived from nonsynonymous mutations are prioritized based on their expression levels as well as their predicted processing and binding affinity to a patient's individual HLA molecules [141]. There are several well-known epitope prediction algorithms, such as NetMHC or NetMHCpan, available in the Immune Epitope Database (IEDB) Analysis Resource (Available from: www.iedb.org) [143–145]. However, growing evidence suggests that predicted binding affinity is not always correlated with ability to trigger T-cell responses. In fact, it has been found that only 1–2 % of putative neoantigens can elicit significant T-cell reactivity [110,146]. This suggests that the current forms of computational neoantigen prediction remain limited in their ability to propose putative neoantigens that are actually translated into peptides, cleaved by the proteasome as predicted, bound to HLA molecules, and presented on the cell surface.

MS-based HLA peptidome analysis could help address the aforementioned problems with computational neoantigen prediction. This modality is able to confirm the *bona fide* epitopes presented by HLA molecules (a later phase of antigen presentation than the HLA-epitope binding). Incorporation of MS analysis is also expected to help reduce rates of false-positive predictions and to avoid the laborious burden of unnecessary validation [15]. As reviewed in detail by Polyakova et al., there are two major distinct workflows of MS to identify neoantigens: a shotgun approach and a targeted proteomics approach [147]. The former is a data-dependent - a mixture of the proteins is digested, separated by liquid chromatography, and analyzed by tandem MS. The latter targets specific peptides, assessing for their presence and quantifying their levels if possible. The former is regarded as more comprehensive and unbiased while the latter is more sensitive [147,148], and various combinations of these approaches are expected to refine the list of neoepitopes predicted by NGS. Indeed, there have been a number of recent studies that support the usefulness of MS for neoantigen identification [149–151].

It is also noteworthy that the results of HLA peptidome analysis frequently contradict the list of epitopes predicted by binding affinity-based analysis [152]. Further complicating the issue, rearrangement of the peptide fragments as a result of proteasomal cis-/trans-splicing has also been described [153]. These findings highlight the limitation of NGS-based *in silico* prediction and underline the significance of MS HLA peptidome analyses. The recently-released NetMHCpan 4.0 [154] and MHCflurry [155] utilize MS-based HLA peptidome data in their training set to refine their *in silico* analysis, offering powerful tools to augment prediction methods.

This is not to say that MS analysis is itself without need for improvement. The sensitivity of MS particularly needs improvement [147]. As mentioned earlier, MS-based HLA peptidome assessment failed to capture a single predicted neoepitope in the APVAC2 workflow of the GAPVAC-101 trial [48]. Of course, absence of evidence is not necessarily evidence of absence; further studies are required to address how to combine and interpret the data inconsistencies between NGS and MS and how to integrate them for neoantigen-targeting therapy in clinical settings.

In addition to MS analysis, a number of novel techniques have been proposed in order to improve the accuracy and the efficiency of *in silico* neoantigen prediction. Łuksza et al. introduced a "neoantigen fitness model" which is based on the relative MHC binding affinity of each neoantigen to its wildtype counterpart as well as a nonlinear dependence on sequence similarity of neoantigens to known antigens [156]. Duan et al. developed another method depending on not only the "differential agretopicity index (DAI)" (which is based on the difference of binding affinity between mutant peptides and their wild-type counterparts) but also the conformational stability of the MHC class I-peptide interaction [157]. Abelin et al. analyzed proteasomal cleavage signatures using HLA peptidome analysis on their HLA class-I monoallelic cell line model [149]. Finally, a novel technique which captures patient-derived T-cells with the ability to recognize the HLA-peptide complexes in the library appears to allow detection of immunogenic epitopes with a low predicted binding affinity [158]. This last finding suggests that epitope selection based simply on a predicted binding affinity threshold should be reconsidered [159]. By addressing all of these issues, more accurate and efficient neoantigen determination will become possible.

4. Concluding remarks

As we have discussed, the paucity of tumor antigens must be overcome to safely and effectively treat GBM and other malignant

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gliomas with low mutation loads. As such, it is crucial to properly understand non-mutant antigens excessively expressed in the tumor, “shared” neoantigens such as IDH1 R132H, H3.3 K27 M, and EGFRvIII, non-shared “private” neoantigens, and virus-derived antigens, and to effectively integrate them as therapeutic targets.

Spatial heterogeneity represents one of the most formidable challenges for immunotherapy of solid cancer [26–30], and multi-layered treatment strategies must be developed. The way forward will require targeting multiple antigens, as well as integrating therapies targeting different immunological hallmarks, such as ICI therapy, vaccines, cell therapies including CAR-T and TCR-T, and other modulators of the immunosuppressive tumor microenvironment [160]. Future studies should also be directed towards refining the antigen selection pipeline, assessing antigen immunogenicity, and developing efficient and reliable manufacturing workflows for vaccines and immune cell therapies.

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Declaration of Competing Interest

Hideho Okada is an inventor of the following US utility patent applications; “H3.3 CTL peptides and uses thereof” (Case Number, SF2015-163), which has been exclusively licensed to Tmunity, Inc., “Anti-EGFRvIII chimeric antigen receptor (Case Number, U Penn 02,980), which has been exclusively licensed to Novartis Pharma, Inc. and “Identification of an IL-13 Receptor Alpha2 Peptide Analogue Capable of Enhancing Stimulation of Glioma-Specific CTL Response” which has been exclusively licensed to Stemline, Inc.

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