Molecular and immunological rationale for the use of tyrosine kinase inhibitors and immune checkpoint inhibitors in glioblastomas

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Glioblastoma (GBM) is the most frequent and invasive tumor of the central nervous system. Maximal surgical resection followed by radiotherapy with concomitant and adjuvant chemotherapy with temozolamide is the standard of care first-line treatment used for GBM. However, increased patient survival based on this first-line treatment is limited, and tumors invariably recur. At recurrence, most common treatment options are further surgical resection, conventional chemotherapy, or the use of the anti-vascular endothelial growth factor (VEGF) agent, bevacizumab. The tumor microenvironment (TME), which is composed of the extracellular matrix, interstitial fluid and stromal cells, including astrocytes, macrophages and endothelial cells, is a key regulator of GBM progression and therapeutic drug resistance. A peculiar feature of the TME in GBM is the blood-brain-barrier (BBB), a semipermeable membrane of endothelial cells connected by tight junctions, capable of preventing the passage of the majority of the pharmaceutical compounds to the GBM tumor. The TME is characterized by an immunosuppressive state with few tumor-infiltrating lymphocytes (TILs) and other cells activating the immune system. The comprehensive characterization of the molecular landscape of somatic genomic alterations of GBM has lead to the identification of a plethora of mutated genes as well as of abnormal rearrangements of several receptors including the epidermal growth factor receptor and platelet derived growth factor receptor α. This has allowed the introduction of novel therapies, including the use of tyrosine kinase inhibitors (TKIs). Moreover, the use of immune checkpoint inhibitors (ICIs) has been successfully introduced in numerous advanced cancers, as well as encouraging results have been obtained that endorse the use of these antibodies in untreated brain metastases from malignant melanoma and from non-small cell lung cancer. Programmed cell death protein (PD-1) receptor/programmed death ligand 1 (PD-L1) inhibitors has been also proposed for GBM treatment. TME, mutational landscape and clonal evolution of GBM tumors are key factors of paramount importance for the efficacy of TKIs and ICIs used in the treatment of GBM. The current review summarizes the principal molecular and TME features of GBM providing the rationale for the use of TKIs and ICI immunotherapy. The main targeted therapies with TKIs and approaches using ICIs, that have been recently proposed, are also discussed.

Keywords

1. Introduction

Glioblastoma (GBM) is the most frequent and invasive tumor of the central nervous system (CNS) [1, 2, 3]. According to the recent update of the World Health Organization (WHO) [2], GBM belongs to the group of diffuse astrocytic and oligodendroglial tumors. Genetic alterations affecting neuroglial stem cells or progenitor cells seem to be involved in GBM pathogenesis [4, 5, 6]. The incidence of this tumor increases with age: median age at diagnosis is 65 years. GBM tumors affect 1.7-fold more often males than females. The WHO subdivides GBM into two major types according to the presence of mutations in the isocitrate dehydrogenase (*IDH*) 1 and *IDH2* genes. GBM with wild type *IDH* accounts for $> 90\%$ of cases [2]. Clinically, the majority of patients present *de novo* grade IV lesions (i.e. primary GBM), while a minority of patients progresses from a less aggressive form of WHO grade II diffuse astrocytomas and WHO grade III anaplastic astrocytomas (i.e. secondary GBM) [2, 7]. Prognosis and age of onset are different between primary GBM and secondary GBM. Primary GBM is typically diagnosed at older age with a worse prognosis in terms of overall survival (OS) [2, 7]. In general, GBM patients display a median OS of about 15 months when undergoing to the canonical first-line treatment consisting of maximal surgical resection, followed by radiotherapy with concomitant and adjuvant chemotherapy, e.g. the oral alkylating agent, temozolomide (TMZ) [3, 8, 9, 10]. The extension of patient survival after TMZ is limited with an average interval of about 2.5 months, and tumors invariably recur [3, 8, 9, 10]. Following the first recurrence, treatment options are represented by further surgical resection when possible, or by conventional chemotherapy, e.g. TMZ (with differ-

ent dosing schedules), nitrosoureas, and by treatment with the anti-vascular endothelial growth factor (VEGF) agent, bevacizumab [11, 12]. However, these treatments have not shown any significant survival improvement in terms of patient survival [9, 10, 13]. The post-bevacizumab progression is often based on bevacizumab plus chemotherapy association, again without significant survival improvement [11, 12, 14]. Recently, the tyrosine kinase inhibitor (TKI) regorafenib has been introduced for the treatment of recurrent GBM [15].

A comprehensive molecular characterization of GBM tumors has allowed the proposal of novel therapies, such as TKIs [16, 17, 18, 19, 20]. Moreover, immune checkpoint inhibitors (ICIs) have been successfully proposed in several cancers including malignant melanoma [21, 22] and non-small-cell lung carcinoma (NSCLC) [23, 24, 25], and encouraging results have emerged for their use in untreated brain metastases of the same tumors [26]. These results have lead to the introduction of programmed cell death protein (PD-1) receptor/programmed death ligand 1 (PD-L1) inhibitors for GBM treatment [27, 28, 29, 30, 31, 32, 33, 34].

In the current review, we summarize the principal molecular and tumor microenvironment (TME) features of GBM providing the rationale for the use of novel targeted therapies and immunotherapy approaches using ICIs for the treatment of GBM-bearing patients. Moreover, the main targeted therapies and approaches using ICIs, that have been recently proposed, are also discussed.

2. Tumor microenvironment

GBM is characterized by a diffuse invasion pattern [35]. Microscopic tumor invasion frequently spreads beyond irradiated regions according to radiotherapy protocols [36]. These infiltrating tumor cells are generally enriched in the stem cell fraction of GBM tumor cells (GSCs) that can make propagation of the tumor easier [37]. The GSCs are characterized by a high refractoriness to chemotherapy, thus driving tumor recurrence and chemoresistance [37]. On the other hand, GBM tumors rarely metastasize in distant organs [38, 39].

The tumor microenvironment (TME) is one of the main actor involved in tumor progression. GBM TME is characterized by the presence of an extracellular matrix (ECM), of an interstitial fluid and of various stromal cells including astrocytes, macrophages and endothelial cells [38, 39]. Peculiar features of the TME in GBM are the blood-brain-barrier (BBB) and the presence of myelinated and interconnected axon tracts as well as specific features of the ECM [38, 39, 40, 41]. Normal brain ECM is enriched in glycoproteins, glycosaminoglycans such as hyaluronic acid and proteoglycans [42, 43]. Hyaluronic acid is mainly localized in the intraparenchymal region, and it is involved in tissue mechanics, organization and hydration. On the other hand, collagen and fibronectin are frequently distributed. In GBM tumors, there is an alteration of the ECM components with a 3-4-fold increment in the presence of glycosaminoglycans with respect to normal tissues. Astrocytes and oligodendrocytes are the major ECM producers in normal tissues. GBM cells can generate a pro-invasive matrix and induce the production of specific ECM components by stromal cells [44]. The BBB is a semipermeable membrane of endothelial cells of the capillary wall connected by tight junctions. While it allows the passage of factors crucial for the neural function, it is capable of preventing the passage of the majority of drugs to the tumor site, thus limiting the achievement of therapeutic drug concentrations [45, 46]. Although during tumor progression the BBB can lose its integrity, it remains impassable for the majority of chemotherapeutic drugs, particularly in the still intact invading tumor regions [47, 48]. Moreover, the presence of interconnected axon tracts represents one of the main limits for surgical resection [40, 49, 50].

GBM tumors are characterized by hypervascularity with an increment in angiogenesis with respect to normal brain tissues. The tumor neo-vasculature is not completely formed, with leaky vessels, augmented interstitial fluid pressure, and hypoxia [51, 52, 53, 54, 55]. GBM tumor cells are capable of invading parenchyma and remodel the surface of myelinated tracks. GBM tumor cells are also capable to rapidly invade vasculature. Invasion of GBM tumor cells can be driven by cellular signaling through the surface receptors CD44 and receptor for hyaluronan-mediated motility (RHAMM) that is activated by hyaluronic acid [56, 57, 58, 59, 60].

The CNS exhibits several peculiar features compatible with the condition of immune-isolation, such as the presence of tight junctions in the BBB and the absence of a classic lymphatic drainage system, nevertheless there is the presence of functional lymphatic vessels, and of different types of antigen-presenting cells (APCs), including microglia, macrophages, astrocytes and canonical APC such as dendritic cells (DCs) [60, 61]. Moreover, activated T cells can invade the CNS. On the other hand, antigens can be presented locally or in the draining cervical lymph nodes (Fig. 1). However, with respect to other tumor types, GBM tumors display low numbers of tumor-infiltrating lymphocytes (TILs), frequently with an exhausted phenotype, as well as low numbers of the other immune stimulative cell types [60]. This reduced quantity and limited activity of T cells in GBM can be ascribed to the peculiar immune environment of the brain [38, 39, 62, 63], with a plethora of immunosuppressive mechanisms at both the molecular and cellular levels [64]. In particular, high levels of the immunosuppressive cytokines transforming growth factor β (TGFβ), interleukin-10 (IL-10) are produced by stromal cells of the brain in response to inflammatory stimuli, such as those from GBM tumor cells [65, 66]. Moreover, indolamine 2,3-dioxygenase (IDO) produced by tumor cells, stimulates the accumulation of regulatory T (Treg) cells. T cell proliferation and function can also be inhibited by microglia and tumor- infiltrating myeloid cells [67, 68].

The process of T cell-mediated immunity is defined by an interplay of stimulatory and inhibitory signals capable of promoting adaptive responses against foreign antigens but also of avoiding autoimmunity. By counteracting activating signaling, immune checkpoints exert a key role in central and peripheral tolerance [69]. In fact, under physiological conditions, immune checkpoint molecules represent a negative feedback to regulate inflammatory responses following T cell activation [70, 71, 72, 73, 74]. The expression of checkpoint molecules, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and PD1, represents a mechanism used by tumors, including GBM, to inhibit and escape the anti-tumor immune response. CTLA-4 is exclusively upregulated in T cells. CTLA-4 is capable of competing with the costimulatory molecule CD28 for the binding of the B7 ligands, with a negative regulatory effect in the early stages of T cell activation. PD-1 belongs to the

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GBM tumor microenvironment
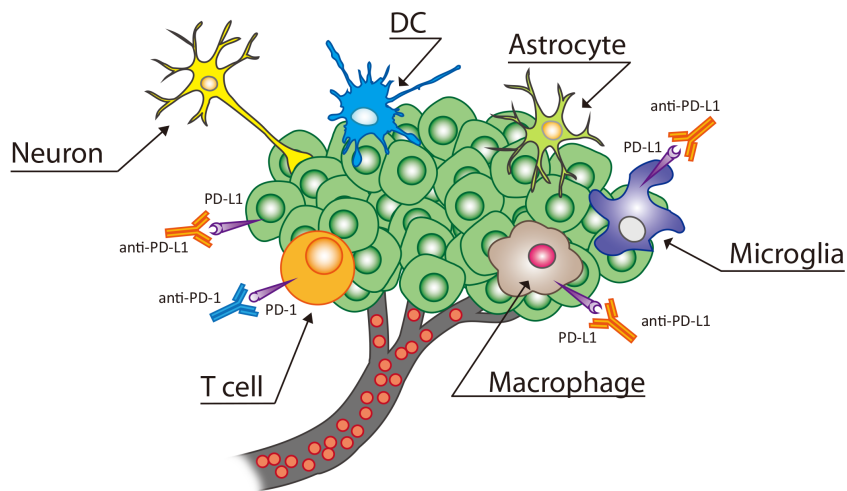
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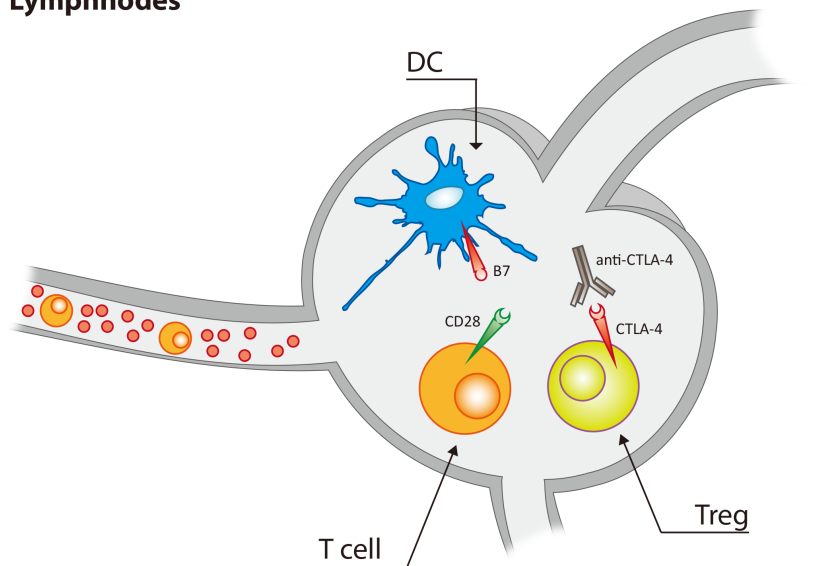
Figure 1. Interactions of immune checkpoint inhibitors (ICIs) in lymph nodes and in the GBM tumor microenvironment.
Cytotoxic T lymphocyte protein 4 (CTLA-4), expressed on the surface of Tregs, is able to inhibit T cells activity by competing with CD28 for the binding of their shared ligands B7-1/2 (CD80/CD86). CTLA-4 blockade mainly acts by targeting CTLA-4-expressing Tregs in lymph nodes. Programmed cell death protein receptor (PD-1)/programmed death ligand 1 (PD-L1) blockade can overcome the T cell exhaustion and reverse the immunosuppression of the tumor microenvironment (TME) by blocking immune checkpoint molecules in the context of the TME of GBM. Abbreviations: Treg, regulatory T cell, DC, dendritic cell, CTLA-4, cytotoxic T lymphocyte protein 4, PD-1, programmed cell death protein receptor; PD-L1, programmed death ligand 1.

B7/CD28 costimulatory receptor family expressed on DCs, natural killer (NK) cells, activated T cells, B cells, and monocytes. The main ligand of PD-1, PD-L1 is expressed on hematopoietic cells, microvascular endothelium cells and parenchyma cells of different organs [74, 75, 76]. PD-1 acts at multiple phases of the immune response modulating T cell activity in the peripheral tissues, including the tumor site [75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85].

3. Mutational landscape and clonal evolution of GBM

In the last years, the genomic landscape of untreated GBM tumors has been investigated. Several genes have been found mutated in GBM including phosphatase and tensin homolog (*PTEN*), tumor suppressor P53 (*TP53*), epidermal growth factor receptor (*EGFR*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), phosphatidylinositol 3-kinase regula-

tory subunit alpha (*PIK3R1*), neurofibromin 1 (*NF1*), retinoblastoma 1 (*RB1*), *IDH1*, platelet derived growth factor receptor alpha (*PDGFRA*), leucine zipper like transcription regulator 1 (*LZTR1*), spectrin alpha, erythrocytic 1 (*SPTA1*), ATRX chromatin remodeler (*ATRX*), gamma-aminobutyric acid receptor subunit alpha-6 (*GABRA6*), kell metallo-endopeptidase (*KEL*), telomerase reverse transcriptase (*TERT*), mutS homolog 2 (*MSH2*), mutS homolog 6 (*MSH6*), mutL homolog 1 (*MLH1*), and PMS1 homolog 2 (*PMS2*) [16, 17, 18, 19, 20, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95]. Moreover, several hotspot mutations have been found, including the *IDH1* R132H mutation, the B-Raf proto-oncogene (*BRAF*) V600E mutation. More than 40% of GBM cases were found to harbor at least one nonsynonimous mutation in genes related to chromatin organization. Of note, these mutations of genes related to chromatin organization were found to be mutually exclusive thus suggesting a biological relevance of chromatin modification in GBM [16, 17, 18, 19, 20, 86, 87].

A definition of a hypermutated profile has been proposed for GBM cases characterized by the presence of mutations in the DNA mismatch repair (MMR) genes, e.g. *MSH2*, *MSH6*, *MLH1*, and *PMS2* [16, 96, 97, 98, 99, 100, 101]. Of note, acquired MMR deficiencies, particularly at the *MSH6* gene, have been more frequently found in GBM cases at recurrence with respect to cases at the first diagnosis, presumably due to the chemotherapeutic treatments. Moreover, an association has been found between a high tumor mutational burden and the loss of MMR protein expression [16, 96, 97, 98, 99].

The *EGFR* gene is one of the most altered genes both at the DNA and RNA levels. Frequently, *EGFR* mutations have been found associated with regional gene amplification [16, 96, 114, 115, 116, 117]. Moreover, a high concordance of mutations has been found between DNA and RNA transcript. Of note, in a relevant proportion of cases the aberrant exon 1-8 junction of epidermal growth factor receptor variant III (*EGFRvIII*) has been found expressed. Additional recurrent non-canonical *EGFR* transcript forms have been also detected [16, 96, 114, 115, 117, 118]. Promoter DNA methylation profiles allowed to separate GBM cases in different clusters according to the DNA methylation status, with different enrichment in classical GBM subtype or mesenchymal GBM subtype. DNA methylation of the O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter is a well-recognized marker of treatment response. In this context, the *MGMT* locus was found methylated in about 50% of GBM cases [16, 96, 114, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128].

The clonal evolution of GBM is complex and treatment options are frequently implicated in the insurgence of a specific genomic alteration and of specific evolutionary patterns of genomic alterations. In particular, treatment failure is frequently associated with intratumoral heterogeneity [16, 17, 96]. Moreover, few driver alterations in GBM tumor cells, through a proliferation phase, seem to lead to a highly differentiated clonal population [14]. Of note, genes known to be implicated in GBM progression, such as *TP53*, *EGFR*, *PDGFRA* are frequently subjected to a process of mutational switching with different mutations of the same gene characterizing diagnosis or relapse [16, 17, 96]. Hypermutated tumors, accounting for 16% of cases, show the highest substitution rates [14]. They harbor mutations in *MMR* pathway genes, in the ma-

jority of the cases in the *MSH6* gene. These *MMR* alterations have been supposed to be associated with putative mutagenic mechanisms of TMZ treatment [16, 17, 96, 97, 98, 99].

Moreover, mutations in the latent transforming growth factor beta binding protein 4 (*LTBP4*) gene, encoding for the LTBP4 protein, capable of binding TGF-β and of activating TGF-β signaling pathway , have been found in 11% of relapse tumors. Of note, the binding of LTBP4 to TGF-β seems to promote tumor growth. A high LTBP4 expression in primary GBM samples with wild-type *IDH1* has been associated with poor survival [16, 17, 96].

Primary GBM tumors more frequently present amplification/mutation of the *EGFR* gene, *PTEN* mutations and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) deletions with respect to secondary GBM tumors [16, 17, 96]. On the other hand, *TP53* mutations, *MGMT* promoter methylation and *IDH1* mutations seem to be more frequent in secondary GBM tumors with respect to primary GBM tumors [16, 17, 96].

4. Mechanisms of chemoresistance

Standard first-line treatment of GBM consists of surgery followed by radiotherapy and chemotherapy with alkylating agents [3, 5, 6]. Alkylating agents such as TMZ but also carmustine (bichloroethyl nitrosurea) and lomustine (chloroethylnitrosourea) can readily cross the BBB and have shown cytotoxic activity by eliciting DNA damage and inducing apoptosis. In this context, MGMT is a DNA repair enzyme that plays a major role in resistance to TMZ and the other alkylating agents by removing the alkyl groups from the O^6 position of guanine [129, 130].

Notably, methylation of the MGMT promoter is associated with its epigenetic silencing resulting in loss of *MGMT* expression [120, 130, 131, 132]. GBM tumors with methylated *MGMT* promoters have been demonstrated to be more sensitive to alkykating agents, whereas GBM tumors with unmethylated *MGMT* promoters express high levels of MGMT and therefore, are more resistant to alkylating agents [122, 130, 133, 134]. A *MGMT* methylated status has been associated with longer progression free survival (PFS) and OS in GBM patients treated with alkylating agents. Moreover, patients with tumors with a methylated *MGMT* promoter have been shown to have survival benefit when treated with TMZ and radiotherapy, compared with those receiving only radiotherapy. On the other hand, patients with GBM tumors with an unmethylated *MGMT* promoter seem to not have any benefit from chemotherapy, regardless of the different administration schedules. This is in keeping with the activity of O^6-methylguanine-DNA methyltransferase as a DNA repair enzyme capable of protecting cancer cells against alkylating agents [16, 96, 114, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 130, 135].

The MMR pathway plays a key role in the modulation of the cytotoxic effect of O^6-methylguanine. The MMR pathway, comprising MLH1, PMS2, MSH2, MSH3, and MSH6 proteins, is involved in the correction of errors in DNA base pairing which arises during DNA replication [135, 136, 137, 138, 139]. Resistance to TMZ can be caused by defects in this pathway that determine a tolerance to the mispairing of O^6-methylguanine with thymine that can occur during DNA replication [135, 136, 137, 138, 139]. This mismatch triggers the MMR-dependent removal of the thymine, and this process can be repeated for various times. These repetitions

can induce DNA double strand breaks with consequent TP53- dependent cell cycle arrest. GBM tumors with alterations in genes of the MMR pathway, such as mutations, exhibit resistance to alkylating agents such as TMZ [135, 136, 137, 139].

The base excision repair (BER) pathway is involved in the repair of the N^7-methylguanine and N^3- methyladenine DNA adducts, that are the most common DNA adducts inflicted by TMZ, accounting for about 90% of all the methylation events [140, 141, 142]. The poly (ADP-ribose) polymerase (PARP-1) enzyme, belonging to the BER pathway, is expressed in GBM tumor cells and it is activated by DNA strand breaks. Inhibition of BER by using PARP inhibitors has been proposed in combination with TMZ treatment [140, 141, 142].

Besides the mechanisms involved in the DNA repair, chemoresistance in GBM can be influenced by the dysregulation of genes/proteins involved in the regulation of apoptosis [143]. Mutations of *TP53*, upregulation of B cell lymphoma -2 (*BCL-2*) and B-cell lymphoma-extra large (*BCL-X_L*) or overexpression of EGFR can disrupt the apoptotic response of GBM cells to DNA damage [143, 144, 145].

TP53 in the wild type mutation status can interact with the promoter of a series of genes including *EGFR*, *MDM2*, *MDM4* and *BCL-2*. TP53 dysregulation in GBM has been associated with BCL-2 upregulation. Moreover, upregulation of BCL-2 expression and EGFR expression has been associated with increased antitumor drug resistance [143, 144, 145].

5. Targeted therapies in GBM

The progresses in the knowledge of GBM-associated molecular signatures have led to the development of new treatment strategies using molecules targeting dysregulated pathways in GBM (Fig. 2).

GBM is a vascularized tumor, characterized by the expression of VEGF and other proangiogenic cytokines influencing tumor cell proliferation, migration and survival [60]. The TKI regorafenib has been approved in the treatment of GBM following the randomized multicentre open label phase 2 trial in which it has been compared with lomustine in patients with a relapsed GBM [15]. In this clinical trial the regorafenib treated GBM group showed a significantly improved OS survival when compared to the lomustine group [15].

Besides regorafenib, other TKIs targeting VEGF family components have been proposed for the treatment of GBM. In particular, cediranib and sunitinib showed promising results in reducing angiogenesis and normalizing vascularization [146, 147].

A targetable pathway in GBM is the PI3K/ mammalian target of rapamycin (mTOR) pathway. In this context, the mTOR inhibitor temsirolimus failed to demonstrate a treatment efficacy as single agent in recurrent GBM [148]. Likewise, the pan-PI3K inhibitor buparlisib failed to demonstrate a treatment efficacy probably due to an insufficient overall PI3K/mTOR pathway inhibition by tolerable doses of buparlisib [149]. mTOR pathway inhibitors also failed to reach efficacy in treatment combinations with radiotherapy and TMZ or in combination with radiotherapy only [150, 151].

The most tested methods to target the TP53 pathway are represented by the neutralization of MDM2 and mouse double minute 4 homolog (MDM4) in GBM patients with a TP53 dysregulation. In fact, several studies have been proposed for GBM cases carrying *MDM2* or *MDM4* gene amplification [152].

In GBM, the RB pathway could be altered for the presence of a *CDKN2A/B* deletion, *CDK4* or *CDK6* amplification or *RB1* gene alterations. In this context, a completed phase II trial using the *CDK4/6* inhibitor palbociclib failed to demonstrate the efficacy of this treatment in GBM [153].

EGFR represents one of the main oncogenes in GBM. EGFR tyrosine kinase inhibitors employed as single agents failed to demonstrate significant activity for GBM treatment [154, 155]. The potential use of MET as target for GBM treatment is still controversial. Several attempts have been made by using the TKIs crizotinib and cabozantinib, resulting in modest efficacy in recurrent GBM [156, 157]. Three different genes encode for neutrophic tyrosine receptor kinases (NTRKs). Larotrectinib and entrectinib have been tested in NTRK fusion-positive GBM, but their efficacy is still to be confirmed [158]. Although fibroblast growth factor receptors (FGFRs) are frequently expressed in GBM, a relevance as potential therapy target seems to be restricted to GBM exhibiting FGFR-transforming acidic coiled-coil containing protein TACC fusions [159]. In this context, the pan-FGFR kinase inhibitor erdafitinib exhibited efficacy with a stable disease and a partial response in two patients with FGFR3-TACC3-positive recurrent GBM [160]. Regarding the possible targeting of the *BRAF*V600E mutations for GBM treatments, a modest treatment efficacy has been obtained in several studies [161]. Finally, eribulin has been proposed as an inhibitor of TERT activity in GBM cases [162].

6. Immunotherapy with ICIs for GBM treatment

Based on the results of using ICIs in other cancers, the use of PD-1/PDL1 inhibitors has been proposed for GBM cases. Clinical trial results have shown that nivolumab, as single agent, does not improve survival compared to bevacizumab in GBM patients with unresectable tumors [29]. Pembrolizumab monotherapy showed limited activity in GBMs with the evidence of only a few objective responses in the context of a compassionate treatment program. Moreover, the addiction of pembrolizumab did not improve the efficacy of bevacizumab monotherapy [29, 30, 31, 32, 33, 34].

The use of nivolumab has been also recently tested in combination treatment regimens with surgery in patients with newly diagnosed or relapsed GBMs [27]. In particular, a single phase II clinical trial was conducted where the use of a pre-surgical dose of nivolumab followed by post-surgical nivolumab was tested in 30 patients, of which 3 were undergoing primary surgery for newly diagnosed GBM. No clinical benefit has been obtained following salvage surgery in the relapsed GBM, whereas 2 of the 3 patients undergoing primary surgery and treated with nivolumab were still alive 33 and 28 months later [27]. Another clinical trial evaluated the use of neoadjuvant and/or adjuvant pembrolizumab in 35 patients with recurrent, surgically resectable GBM tumors. In this context, the addiction of the neoadjuvant treatment with pembrolizumab to the surgery and the adjuvant treatment had significantly increased OS of GBM patients [28].

Mutations in the *PTEN* gene have been found to be enriched in GBM patients who are not responsive to ICIs [163]. The presence of *PTEN* mutations has been associated with an immunosuppres-
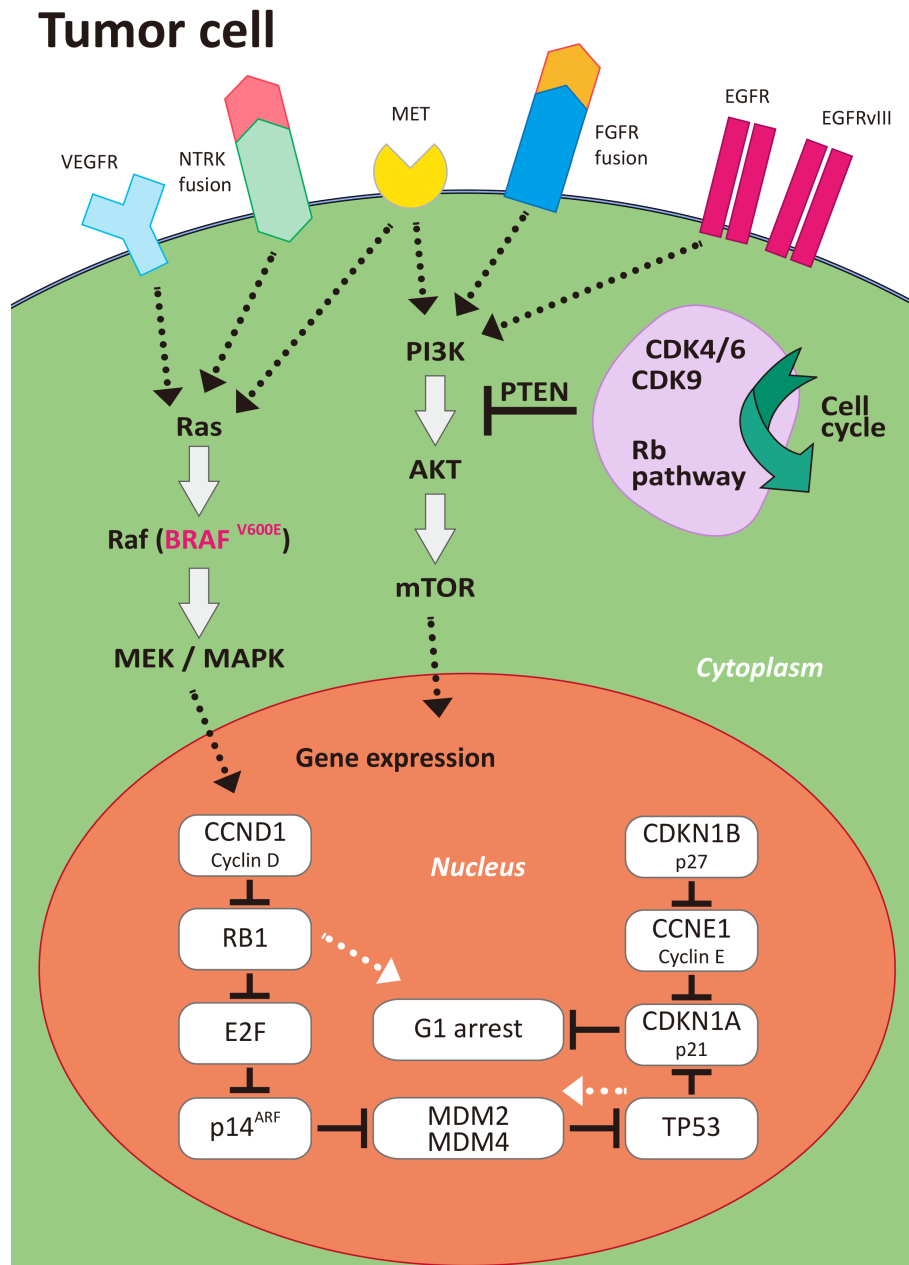
Figure 2. Candidate molecular pathways for targeted therapies in GBM. The introduction of next generation sequencing methods has led to the identification of specific molecular signatures in GBM. This detailed characterization of the GBM-associated molecular signatures has allowed a more personalized therapeutic approach with the development of novel therapies including those employing tyrosine kinase inhibitors (TKIs). Abbreviations: VEGFR, vascular endothelial growth factor receptor; NTRK, neutrophic tyrosine receptor kinases; MET, MET proto-oncogene; FGFR, fibroblast growth factor receptor; EGFR, epidermal growth factor receptor, EGFRvIII, epidermal growth factor receptor variant III; Ras, RAS protein; BRAF, B-Raf proto-oncogene; Raf, Raf protein; MEK/MAPK MAPK/ERK kinase, mitogen-activated protein kinase, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; AKT, AKT Serine/Threonine Kinase 1; mTOR, mammalian target of rapamycin; PTEN, phosphatase and tensin homolog; CDK4/6; CDK9, CDK4/6; CDK9, cyclin dependent kinases 4/6, 9; Rb, retinoblastoma, CCND1, cyclin D1; E2F, E2F transcription factor, MDM2, mouse double minute 2 homolog; MDM4, mouse double minute 4 homolog; CDKN1B, cyclin-dependent kinase inhibitor 1B; CCNE1, cyclin E1; CDKN1B, cyclin-dependent kinase inhibitor 1A; TP53, tumor protein TP53.

sive TME [129, 164, 165].

Mutations of *BRAF*/protein tyrosine phosphatase non-receptor type 11 (*PTPN11*) were found to be enriched in tumors which are responsive to ICIs. In this context, given that mitogen-activated protein kinase (MAPK) pathway inhibition can significantly increase the efficacy of immunotherapy, a combination treatment of ICIs and MAPK inhibitors could be appropriate in GBM patients with *BRAF/PTEN11* mutations [166, 167].

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Regarding the lack of responsiveness to ICIs, it has been shown that GBM cases which are not responsive to ICIs, are characterized by an enriched expression of genes associated with immunosuppression prior to the initiation of ICI treatment, whereas in GBM cases responsive to ICIs, the acquisition of an immunosuppressive condition seems to occur post-treatment [17, 163]. This could be associated with an intrinsic resistance in non-responsive patients and in an acquired resistance by selective pressure in GBM tumors responsive to ICIs [17, 163].

The use of the neoadjuvant antitumor agents nivolumab or pembrolizumab has been associated with an enhanced expression of chemokine transcripts, and a higher immune cell infiltration. Moreover, neoadjuvant administration of anti-PD-1 antibodies has been associated with a functional activation of TILs eliciting an interferon response within the TME. This T- cell-mediated interferon response seems to be related to a downregulation of the expression of cell cycle related genes with a decrease in tumor cell proliferation [27, 28]. An increase in T cell receptor (TCR) clonal diversity among tumor-infiltrating T lymphocytes has also been detected following the treatment with neoadjuvant nivolumab. Treatment with nivolumab was also associated with long complementarity-determining region (CDR3) of TCR when compared with cases not treated with ICIs. Moreover, it has been suggested that treatment with nivolumab could prevent reduction of both adaptive and myeloid immune cell populations [27, 28].

7. Proposed biomarkers for responsiveness to ICI treatment

The use of ICIs has demonstrated heterogeneous responses in GBM cases both in the clinical practice and in clinical trials, defining the need of identifying useful predictive biomarker of responsiveness. The first marker evaluated as predictor of a clinical response to ICIs was PD-L1 expression [168], that was correlated with specific histological and molecular features, demonstrating a possible correlation with *IDH* status. Specifically, a higher PD-L1 expression in gliomas has been related with a wild type *IDH* status, when compared with cases with an *IDH* mutated status, thus indicating a potentially higher responsiveness to ICIs in wild type *IDH* cases [169, 170, 171]. Of note, high PD-L1 expression have been found in mesenchymal GBM, thus suggesting an association with the aggressiveness of the tumors [172]. More recently, the tumor mutational load has been evaluated as a predictive marker of responsiveness to ICIs. In particular, a high mutational load could be associated with a higher presence of mutation-associated neo-antigens (MANAs) putatively capable of stimulating specific T cell clones, with a consequent increase in tumor immunogenicity. However, a putative cut-off to identify responsive cases seems to differ among the different cancer types. Moreover, a standardization of the protocol to determine the tumor mutational burden as well as of the adopted techniques for this determination has not yet been proposed. Evaluation of tumor mutational burden by whole genome sequencing has not been generally demonstrated to sufficiently predict long term clinical benefits [173, 174, 175, 176]. Moreover, in recent studies higher somatic mutations and neoepitope loads have not been found in GBM cases responsive to ICIs [163]. On the other hand, a low mutational load does not appear to preclude the infiltration into the tumor of T cells responsive to specific MANAs, also in the context of the immunosuppressive TME characterizing GBM tumors [27, 28, 163]. Another proposed biomarker is the presence of *MMR* gene abnormalities, that has been related with a clinical response to ICIs in several clinical trials including in GBM patients [27, 28]. An additional feature proposed as a biomarker of responsiveness to ICI is the expression of MHC class I molecules that has been found highly heterogeneous in GBM, with a higher expression in more responsive GBM cases [177].

8. CAR-T cell therapy for GBM treatment

The success of chimeric antigen receptor -T (CAR-T) cell therapy in hematological malignancies, with CAR-T cells targeting CD19 approved for B cell acute leukemia and lymphomas [178, 179], has favored the introduction of this therapy approach also in solid tumors including GBM. In particular, for GBM treatment, CAR-T cells have been engineered mainly to target the following antigens EGFRvIII, human epidermal growth factor receptor 2, (HER2) and IL-13 receptor α2 (IL-13Rα2), for which clinical trials have been proposed. Results of the proposed clinical trials showed that the employment of CAR-T cell therapy for GBM is feasible, safe and potentially efficacious, although, as for other solid tumors, there are still several substantial obstacles [180, 181, 182]. In particular, the major challenges include tumor heterogeneity in terms of antigen expression, access of CAR-T cells to the tumor site as well as resistance of the TME to CAR-T therapy [183, 184, 185, 186]. To overcome both antigen heterogeneity and antigen loss, one approach is to simultaneously target more than one tumor associated antigen with multi-specific CAR-T cells. In GBM tumors, there are several obstacles that a CAR-T cell must overcome to reach the tumor site, including abnormal vasculature capable to block T cell entry. One practical approach is represented by intracranial administration showing some promising results for anti-IL13Ra2 [181, 182]. Different approaches have been introduced to overcome TME immune suppression in the context of CAR-T cell therapy. In particular, the concomitant use of ICIs has been proposed. Other strategies are represented by the introduction of several CAR-T modifications such as the knocking out of genes encoding T cell inhibitory receptors or signaling molecules (e.g. PD-1 or CTLA-4), or the co-expression of activating chimeric switch receptor (CSR), that combines the extracellular domain of an inhibitory receptor (PD-1 or CTLA-4) linked with the cytoplasmic co-stimulatory signaling domain of CD28 (Fig. 3) [187, 188, 189, 190, 191].

9. Conclusions

In the last ten years, comprehensive genomic analyses have revealed that GBM tumors are highly heterogeneous with different tumor subgroups characterized by specific molecular features [16, 17, 96]. The high degree of heterogeneity makes tumor classification difficult as well as the designing of effective customized therapies capable of targeting dysregulated pathways. Moreover, the molecular pathways that can be targeted are often functionally synergic making the inhibition of a single particular molecular mechanism frequently useless [16, 17, 96]. In this context, a relevant role is also carried out by clonal selection that allows the propagation of drug resistant clones to a specific targeted ther-
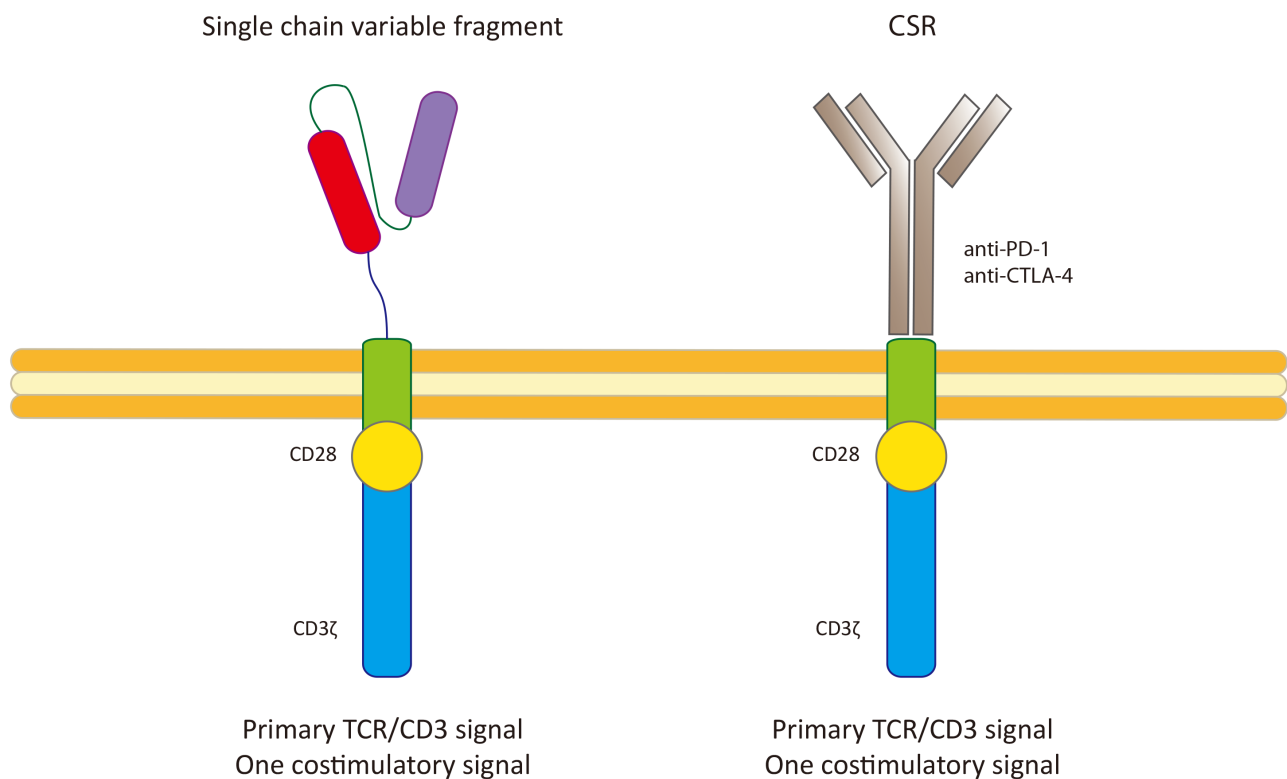
Figure 3. Modified CAR-T cells to counteract immunosuppressive tumor microenvironment (TME). A strategy to improve chimeric antigen redirected T (CAR-T) cell efficacy in solid tumors including GBM is represented by the co-expression of an activating chimeric switch receptor (CSR), that combines the extracellular ligand-binding domain of an inhibitory receptor (PD-1 or CTLA-4) fused through a transmembrane domain with the cytoplasmic co-stimulatory signaling domain of CD28. The engagement of the CSR allows the transmission of an activating signal instead of the normal physiological inhibitory signal. Abbreviations: CSR, chimeric switch receptor; TCR, T cell receptor; PD-1, programmed cell death protein 1; CTLA-4, cytotoxic T-lymphocyte antigen 4.

apy due to the presence of specific genomic alterations or pathway activations/dysregulations [16, 17, 96]. Another possible reason for the failure of targeted therapies is that several genomic alterations can drive only the early stages of progression, whereas their role is overridden in the later stages by other molecular mechanisms. Another relevant obstacle to an effective therapy is represented by the BBB which affects the targeting of chemotherapeutic drugs to the GBM tumor [45, 46]. The TME can cause chemoresistance being capable of promoting tumor cell proliferation and of selecting aggressive cancer cells including GSCs. In a vicious circle, the interaction of TME with GSCs can further increase chemoresistance. The TME of GBM is largely immunosuppressive, this condition can strongly affect efficiency of ICI treatments [35, 36, 50, 51, 52, 53, 54, 55, 56, 57, 58]. Moreover, chemotherapeutic treatments can cause a reduction in the levels of circulating $CD4^+$ and $CD8^+$ lymphocytes [192, 193]. On the other hand, the identification of immunological signatures capable to predict the responsiveness to ICIs is still an important clinical need [24, 25, 26, 27, 28, 29, 30, 31].

10. Future perspectives

GBM remains an incurable and lethal disease despite the continuous attempts to increase the survival of GBM affected cases. Although TKI use has been so far associated with a limited re-

sponse in terms of survival increases of GBM treated cases, further efforts could be made in the definition of combination treatment approaches to include their use in the canonical well-established therapeutic modalities.

The data collected so far regarding the ICI employment in GBMs are modest and still incomplete to propose them as a standard therapeutic approach for GBM affected patients [24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34] . However, results of the use of nivolumab and pembrolizumab administered as adjuvant and neoadjuvant treatments in the context of chemo-radio immuno-combinations seem to be more promising, at least for a certain fraction of patients. Several candidate biomarkers have been proposed to predict responsiveness to ICI treatment for GBM patients. Nevertheless, a strong correlation has not yet been found between the proposed biomarkers and clinical and radiological response to ICIs. In this context, further analyses remain necessary within both pre-clinical and clinical studies regarding different aspects encompassing somatic features of tumor cells, mutational landscapes, deficiency in DNA mismatch repair, transcription factors, immune-related gene expression miRNA signatures, and association with neoantigens. Additional information could be obtained by using computational/mathematical models useful to reach a better understanding of the molecular complexity generated by the differences in genomic, transcriptomic and immune-related fea-

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tures.

An ever increasing knowledge of this molecular complexity could also provide the rationale for the introduction of the use of CAR-T cells, in combination with ICIs or TKIs, in the treatment paradigm of GBM.

Conflict of interest

There are no conflicts of interest to disclose.

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