Hedgehog signaling regulates the development and treatment of glioblastoma (Review)

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Abstract. Glioblastoma (GBM) is the most common and fatal malignant tumor type of the central nervous system. GBM affects public health and it is important to identify biomarkers to improve diagnosis, reduce drug resistance and improve prognosis (e.g., personalized targeted therapies). Hedgehog (HH) signaling has an important role in embryonic development, tissue regeneration and stem cell renewal. A large amount of evidence indicates that both normative and non-normative HH signals have an important role in GBM. The present study reviewed the role of the HH signaling pathway in the occurrence and progression of GBM. Furthermore, the effectiveness of drugs that target different components of the HH pathway was also examined. The HH pathway has an important role in reversing drug resistance after GBM conventional treatment. The present review highlighted the relevance of HH signaling in GBM and outlined that this pathway has a key role in the occurrence, development and treatment of GBM.

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Abbreviations: GBM, glioblastoma; CNS, central nervous system; HH, Hedgehog; PCNS, primary central nervous system; GLI, glioma-associated oncogenes; CDK4, cyclin-dependent kinase 4; SHH, Sonic HH; PC, primary cilia; SUFU, suppressor of fused homolog; KIF7, kinesin 7; SMO, smoothened; PKA, protein kinase A; GSK3, glycogen synthase kinase 3; CK1, casein kinase 1; EMT, epithelial-mesenchymal transition; CAFs, cancer-associated fibroblasts; MMP2, matrix metalloproteinase-2; FLT1, Fms-related tyrosine kinase 1; HHIP, HH-interacting protein; mTORC1, mammalian target of rapamycin complex 1; TMZ, temozolomide

Key words: Hedgehog, glioblastoma, Sonic, patched-1, smoothened, drug resistance, therapeutics

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1. Introduction

Primary central nervous system (PCNS) tumors account for 12% of all neoplasms (1). Glioblastoma (GBM) is the most common type of primary malignant CNS tumor, representing ~48% of all primary malignant CNS tumors and 57% of all gliomas (2). In spite of the progress made in the treatment of GBM in recent years (including surgery, radiotherapy, chemotherapy and targeted therapy), the overall prognosis is still not ideal and the long-term survival rate is low. Certain studies indicated that teenagers and young adults account for 27% of all PCNS tumor cases and the average age was 29 years (1,3). If patients are diagnosed with cancer in those busy years of their life, this may take a serious toll on both their body and mind, and in turn on their spouses and offspring (4). Upon diagnosis, the prognosis of GBM is poor, with months to a year left to live, so that this may also have detrimental effects on the patients' dependents and family (4).

In recent years, important advances have been made in the exploration of the molecular pathogenesis of tumorigenesis and progression, but this has not been applied to significantly improve patient prognosis. It is thus essential to identify biomarkers for diagnosis, as well as means of reducing drug resistance and delivering treatments, including personalized targeted therapies in the study of GBM. The role of Hedgehog (HH) signaling in the pathophysiology of GBM is underscored by a growing number of publications (5-7). The HH pathway is increasingly being revealed to have an important role in the growth, progression, prognosis and treatment of GBM (8-10).

The present review will discuss the contribution of HH signaling in the development and treatment of GBM. Chemotherapy, targeted therapy and radiotherapy in the HH pathway will also be discussed and the issue of improving partial drug resistance through this pathway will be addressed.

2. Overview of GBM

GBM originates from the glial stem or progenitor cells and is characterized by molecular heterogeneity, with a mean survival of only 15 months after diagnosis (11). Commonly mutated genes and core pathways in sporadic GBM were identified based on molecular mapping and three major GBM subpopulations were identified in combination with other dimensions (gene expression, DNA methylation). The DNA methylated α group amplified cyclin-dependent kinase (CDK)4 and platelet-derived growth factor in the three ways (classical gene expression; classical like; receptor tyrosine kinase II). High-frequency amplification of EGFR and homozygous deletion of CDK inhibitor 2A/B occurred in the DNA methylation group. Mesenchymal/mesenchymal subtypes are abundant in tumors with loss of neurofibromatosis type 1 and increased tumor macrophage infiltration (12,13). The above three types are the most common types of GBM and all involve mutations in telomerase reverse transcriptase promoters (14,15). In addition, characteristic epigenetic patterns are associated with certain putative driving mutations that are important in GBM, according to recent studies (16,17). Examples include mutated isocitrate dehydrogenase (IDH)1 and IDH2, H3.3 histone A or H3 clustered histone 2 mutations, particularly H3K27M in diffuse midline glioma and H3G34R/H3G34V mutations in young patients with GBM (16,17). However, their clinical implication for these GBM subtypes has not been proven. These studies indicate that different subtypes of GBM are caused by different oncogenes, which paves the way for the exploration of highly specific personalized targets.

GBM is characterized by continuous vascularization, tissue invasion and metastasis, metabolic recombination or alteration, immune regulation and promotion of the tumor microenvironment. All of the above characteristics lead to high resistance of GBM to radiotherapy and chemotherapy, which brings a non-negligible challenge to the treatment of the disease (18).

GBM has different subtypes, but the current international treatment methods mainly include chemotherapy [such as temozolomide (TMZ)], radiotherapy (RT) and surgical treatment. Monotherapy may be well tolerated in elderly patients (>65 years) with poor functional status. It has been reported that low-grade RT (40 Gy/15 doses of 2.67 Gy over 3 weeks) was higher than the standard 60 Gy for 6 weeks (19,20). Relapsing patients may be treated with surgery (as palliative care only) or other options include TMZ reactivation, nutrition and bevacizumab. However, there is no specific clinical evidence of prolonged survival (21,22). Several valuable clinical trials are under development for the treatment of GBM, including targeted molecular (precise) therapies (targeting gene mutations and associated signaling pathways, DNA damage repair, tumor metabolism), checkpoint inhibitors/immunomodulation agents and viral therapies. Despite the GBM treatment options available, metastatic disease remains a great concern. Therefore, it is of marked importance to find novel therapeutic targets and new drugs targeting the HH signaling pathway to regulate the occurrence, development, treatment and chemotherapy resistance of GBM (23,24).

3. The HH signaling pathway

HH is a morphogenetic gene, which is highly conserved from drosophila to humans. The HH signaling pathway has an important role in embryonic development, cell proliferation, differentiation and maintenance of tissue polarity (25). Inactivation of this pathway during development may lead to congenital defects, while over-activation in adults is related to tumorigenesis (26,27). The HH protein family includes Sonic HH (SHH), Indian HH (IHH) and desert HH (DHH) (28). In mammals, the mechanism of HH signaling is complex and occurs in primary cilia (PC) (29). In PC, HH protein binds to 12 transmembranes (TM) receptors [Patch1 (PTCH1) and PTCH2] to activate pathways, so that 7-TM protein smoothened (SMO) is inhibited (30). The HH signal is transmitted downstream of SMO through the complex composed of kinesin 7 (KIF7), suppressor of fused homolog (SUFU) and full-length glioma-associated oncogenes (GliFL), which promotes the dissociation of SUFU from GLI protein and then releases transcription factors (GLI1, GLI2 and GLI3) (31,32). GLI2 and GLI3 constitute GliFL, which act as both GLI activators (GLIA) and GLI inhibitors (GLIR) (33,34). After activation of SMO, GLI2/3 P1-6 clusters were dephosphorylated and separated from SUFU (35), which facilitates the transfer of GLIA into the nucleus and the initiation of the transcription of target genes, and their pathway genes (PTCH1, GLI1) (36,37). GLI1 is the main HH target gene and its expression further promotes the activation of the HH signaling pathway at the transcriptional level (38). KIF7, in turn, coordinates HH signaling at the top of the PC and avoids GLI3 from cracking into an inhibited form in response to HH (39). This GLI transcription factor signal transduction pathway is the canonical HH signaling pathway (Fig. 1A).

When HH ligand is absent, PTCH inhibits the activity of SMO by inhibiting the translocation of SMO in PC (40). GLIFL is phosphorylated by protein kinase A (PKA), glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1), and then recognized by β-trCP and cleaved into GLIR (41,42). This results in the proteolytic cleavage of GLIFL into the form of a C-terminal truncated repressor known as a GLIR (33). SUFU is a negative regulator that binds to GLI proteins and prevents them from migrating to the nucleus (43). GLIR enters the nucleus, binds to HH target gene promoters and inhibits their expression (Fig. 1B).

HH signaling, canonical and non-canonical signaling, exist in parallel, and the mechanisms are complex. Non-canonical HH signaling is the most common HH-dependent reaction process, independent of GLI transcription factors or PC (44). Non-canonical HH signaling pathways may be divided into type I (independent of SMO) and type II (dependent on SMO) (45).

The canonical HH pathway is related to tumorigenesis and detransformation development. In adults, this signal abnormality has a key role in promoting the proliferation and differentiation of numerous tumor types. Its carcinogenic

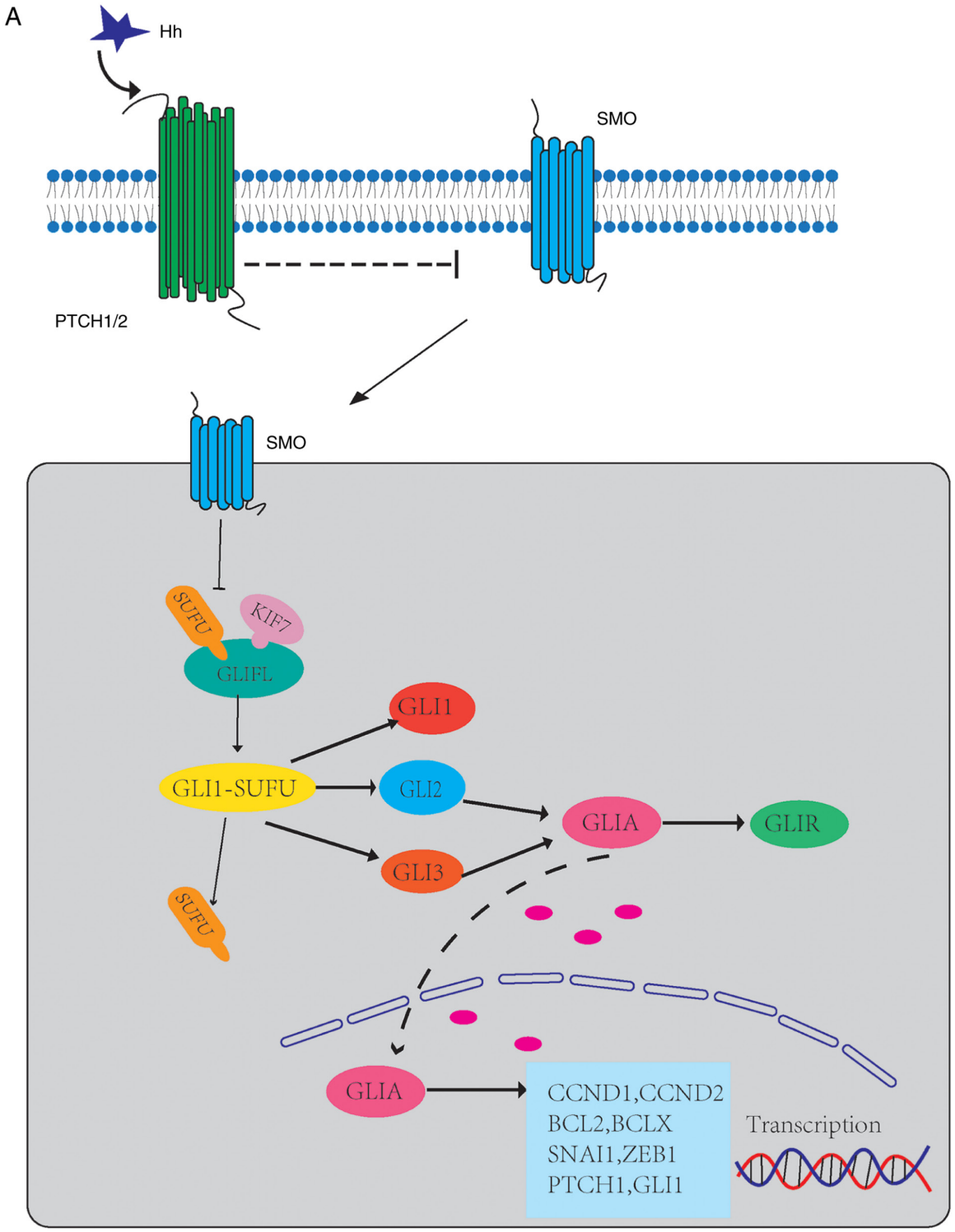

Figure 1. Continued.

mechanisms mainly include abnormal cell differentiation, neovascularization, epithelial-mesenchymal transition (EMT) and enhanced invasiveness (46-48). Initially, HH signaling was mainly studied in brain cancer, skeletal muscle and skin cancer (49-51). However, in recent years, studies have indicated that this pathway is abnormal in numerous tumor types, including stomach, pancreas, lung and breast tumors (52-54). As HH signaling is activated in various types of cancer and contributes to cancer proliferation, progression and invasiveness, the HH signaling pathway is anticipated to provide new targets for cancer therapy.

4. Molecular mechanisms of the HH signaling pathway in GBM

HH signaling pathway and GBM microenvironment. The tumor microenvironment/stroma is closely related to tumorigenesis, metastasis and invasion (55,56). The tumor

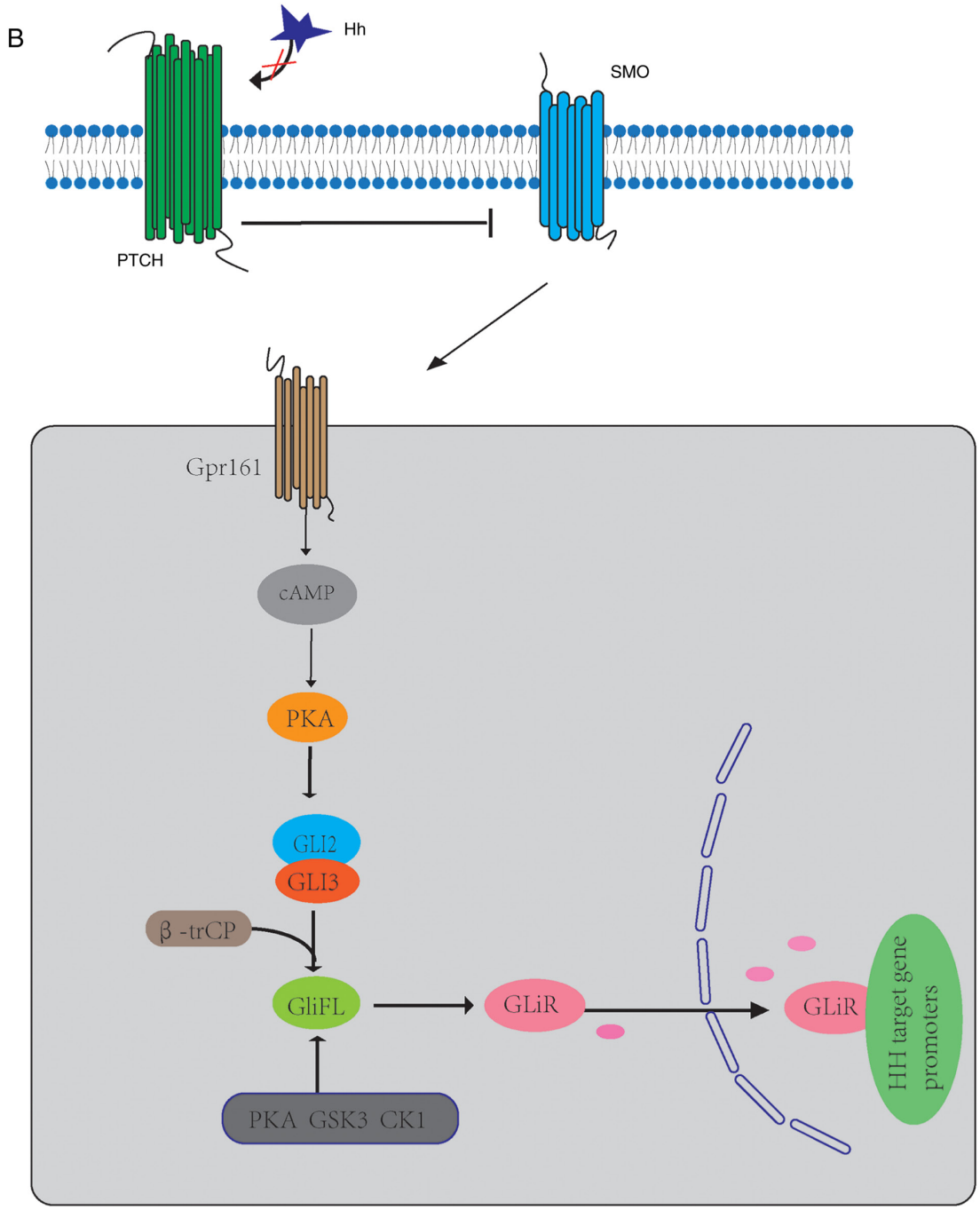

Figure 1. Schematics of the mechanisms of HH signaling in GBM. (A) The HH protein activates a signaling cascade by binding to the 12-TM receptors PTCH1 and PTCH2 and leading to derepression of the seven-TM protein SMO. The HH signaling may now proceed downstream of SMO via a cytoplasmic protein complex consisting of Kif7, SUFU and GLIFL. When the signal reaches SUFU, the GLI1-SUFU complex dissociates, allowing it to release transcription factors (GLI1, GLI2 and GLI3). GLI2 and GLI3 are constitutively expressed and serve as transcriptional activators, GLIA, in their full-length form and as transcriptional repressors, GLIR, after partial proteasomal processing. Activation of SMO leads to the dephosphorylation of GLI2/3 P1-6 clusters and their dissociation from SUFU, which facilitates the transfer of GLIA into the nucleus and the initiation of transcription of target genes. (B) In the absence of the HH ligand, Ptch represses the activity of SMO by inhibiting its translocation into the PC. Gpr161 localizes to the PC to maintain high CAMP levels and PKA activity, which phosphorylates P1-6 clusters located on GLI2/3. Subsequently, GliFL is phosphorylated by PKA, GSK3 and CK1 and recognized by β-trCP. This results in the proteolytic cleavage of GliFL into the form of a C-terminal truncated repressor known as a GLiR. GLiR is translocated to the nucleus, where it binds to HH target gene promoters and inhibits their expression. HH, hedgehog; SMO, smoothened; PTCH, patched; TM, transmembrane; Kif7, kinesin family member protein 7; SUFU, suppressor of fused; GPR161, G-protein coupled receptor 61; CAMP, cyclic adenosine monophosphate; PKA, protein kinase A; GliFL, full-length glioma-associated oncogene; GSK3, glycogen synthase kinase-3; CK1, casein kinase 1; GLIR, GLI repressor; GLIA, GLI activator; PC, primary cilia.

microenvironment/stroma is mainly composed of endothelial cells, adipocytes, immune cells and cancer-associated fibroblasts (CAFs) (57). CAFs are able to secrete soluble factors to stimulate cancer cells, thereby triggering tumor metastasis and chemotherapy resistance (58-60). Recombinant human Sonic HH N-terminal peptide (rhSHH) enhances HH signaling, accompanied by increased mRNA and protein levels of matrix metalloproteinase-2 (MMP2) and MMP9. Furthermore, the

protein expression of GLI1 was positively associated with the protein expression of MMP-2 and -9, which promoted the adhesion and invasion of GBM cells (60). It has been reported that gap junctions have a role in tumor growth and progression. Torrisi *et al* (61) modulated SHH signaling and connexin 43 (CX43)-based intercellular communication in an *in vitro* model. Modulation of SMO with the use of a known agonist (i.e., taxamine) and a known antagonist (i.e., cyclodopamine) affected CX43 expression levels and thus affected related functions. In addition, SMO activation also promoted cell proliferation and migration. Of note, inhibition of the CX43 channel prevented the SMO-induced effects (61).

Therefore, further exploration of the mechanisms of the HH signaling pathway in the tumor microenvironment may lead to better targeting of this pathway to fight cancer.

Regulatory mechanism and role of SHH in GBM cells. In the development of GBM disease, PC serve as cell antennae to transmit and regulate a variety of signaling pathways and SHH is one of the most important pathways. SHH levels are significantly increased in GBM cells compared with normal brain tissue and SHH overexpression induced neuroectodermal angiogenesis during mouse embryonic development (62-64). A study has indicated that Fms-related tyrosine kinase 1 (FLT1) is significantly increased in GBM cells and overexpression of FLT1 increased the expression of SHH in cells (64). Knockdown of SHH reduced the migration and invasion mediated by FLT1 overexpression, while overexpression of SHH restored the migration and invasive ability of FLT1 knockdown (64). FLT1 is a tyrosine kinase receptor that binds VEGF-A with several times the affinity of other kinases inserted into domain receptors and has been reported to promote tumor growth and metastasis (65). VEGF-A is one of the key factors promoting tumor angiogenesis and activation of the VEGF-A pathway requires the binding of VEGF-A to its receptor FLT1 to generate downstream signals to stimulate the proliferation and development of tumor cells and provide tumor blood vessels for the growth and metastasis of GBM (64,66). In addition, brain tumorigenic-initiating cells produce DHH ligands to realize the paracrine DHH/PTCH2 signaling cascade, transmit high permeability and angiogenesis, and also promote GBM growth (6). Chen *et al* (67) reported that C-terminal binding protein 2 (CtBP2) expression was increased and zinc finger and BTB domain containing 18 (ZBTB18) expression was decreased in GBM tissues, and the two were negatively correlated. CtBP2 short hairpin (sh)RNA interacts with ZBTB18 to block cells in G0/G1 phase, inhibit the SHH-Gli1 pathway and reduce the tumor volume (67). However, whether this effect is exerted by increasing SHH gene expression has remained to be elucidated. Therefore, targeted FLT1 or CtBP2 therapy may be a promising direction to develop anti-metastasis agents.

GBM develops through a complex interlocking signaling pathway. RhSHH enhances the HH signaling pathway, which increases the production of MMP-2 and -9 through the PI3K/AKT pathway, thereby regulating migration and invasion of basal membrane cells and promoting GBM cell adhesion, invasion and migration (60). By contrast, triggering the vasoactive intestinal peptide receptor system is triggered to reduce GBM cell migration and invasion through PKA-dependent PI3K/AKT and SHH/GLI1 pathway blocking (68). Similarly, Henao-Restrepo *et al* (69) reported that PI3K/AKT/mammalian target of rapamycin complex 1 (mTORC1) and SHH/GLI signaling pathway proteins were expressed differently in human gliomas with different tumor types and grades, suggesting that the activation of these signaling networks is related to the occurrence and development of high-grade gliomas.

Multiple studies have indicated that the SHH signaling pathway promotes the plasticity of cancer cells by regulating the adhesion between cells and the extracellular matrix, thus increasing the motility and aggressiveness of cells, leading to poor prognosis of patients (60,65,67,68). Statistical analysis of the The Cancer Genome Atlas (TCGA) dataset (TCGA-Glioblastoma June 2016) suggested that SHH upregulation was associated with decreased overall survival (64).

Hedgehog-interacting protein (HHIP), which is located on chromosome 4q31.21-31.3, is defined as an antagonist of SHH, IHH and DHH. Chang *et al* (9) were the first to demonstrate that the expression of HHIP determined by immunohistochemistry is an independent prognostic marker of favorable outcomes in patients with GBM.

Expression and role of GLI1 in GBM. Although GLI1 was originally identified as the amplified gene in malignant human gliomas (70), GLI1 amplification is infrequent in most cancers such as GBM (71). However, since GLI1 is a vital downstream target of the HH pathway, the mRNA level of GLI1 is a reliable indicator of HH pathway activity, this suggests that control of GLI1 protein conversion is critical for GLI-dependent transcription and regulation of the HH signaling pathway (53). And GLI1 protein levels are upregulated in a variety of cancers, and high levels of GLI1 are often associated with tumor progression (72,73). Low GLI1 mRNA levels were similarly negatively correlated with survival in patients with GBM. GLI1 mRNA expression in GBM was significantly lower than in patients with high-HH-medulloblastoma (MB) but significantly higher than in patients with low-HH-MB, and GLI1 mRNA expression is a single continuous distribution, rather than being discrete high/low clusters (5,74). GLI1 promotes the nuclear import of GLI1 into GBM multiforme cells through its transcription factor Forkhead box M1 (FOXM1), thereby increasing the expression of its target genes (75).

Zhou *et al* (73) reported that in GBM cells, ubiquitin specific peptidase 48 (USP48) gene knockout inhibited cell proliferation and downstream GLI1 target gene expression, thereby inhibiting glioblastoma by USP48 removing ubiquitin-binding compounds on GLI1 and thereby inhibiting GLI1-dependent proteasome degradation. In addition, to a certain extent, GLI1 determines the effect of USP48 on cell proliferation and tumorigenesis and the HH pathway also induces USP48 expression via GLI1 trans-activation, thus forming a mutual feedback loop (73). Similarly, Chang *et al* (8) indicated that Engraile 1 (EN1) was highly expressed in GBM cells and tissues and positively regulated GLI1 levels. In addition, EN1 also affected HH signal transduction by regulating PC length and the PC transport-related protein TUB-like protein 3, a PC transport-related protein, to control the proliferation, colony formation, migration and tumorigenesis of GBM cells *in vivo*. Truncated GLI1 (TGLI1) acted as a functionally enhanced GLI1 with an enhanced ability to promote angiogenic heparanase expression. *In vivo* and *in vitro*, TGLI1 is more

likely to promote GBM angiogenesis and growth than GLI1. Therefore, TGLI1 is a novel mediator promoting GBM angiogenesis through the HH signaling pathway and heparinase is a novel transcriptional target of TGLI1, providing new clues for molecular pathways of tumor angiogenesis and invasive growth (76).

It was observed that both the activation of metabolic glutamate receptor subtype 4 and naringin are able to inhibit the expression of GLI-1 in cells and affect HH signaling pathway transduction, thus inhibiting cell proliferation and promoting cell apoptosis to inhibit the growth of GBM cells (77,78). These may be potential drug targets for controlling GBM cell growth by blocking HH signaling.

Expression and role of GLI2 in GBM. Molecular crosstalk is present between mTORC1/2 and HH pathway activity (71,79,80). In GBM, higher mTORC2 activity enhances the expression of several HH pathway molecules (GLI1, GLI2 and PTCH1). A further study by Maiti *et al* (80) indicated that mTORC2 inhibits GLI2 ubiquitination by inactivating GSK3β, thereby promoting GLI2 stability and nuclear translocation, then modulating the role of HH pathway activity in GBM angiogenesis, metastasis, cell proliferation and cancer stem cell (CSC) regeneration. In addition to influencing mTORC1/2 and HH pathway interactions, GLI2 also affects HH and Wnt pathways and has an important role in GBM stem cell (GSC) maintenance. GLI2 knockdown using lentiviral-mediated shRNA downregulated HH-related and Wnt signaling pathway-related genes, including leucine-rich repeat-containing G-protein coupled receptor 5, inhibited tumor cell proliferation and invasive capacity, and induced apoptosis (81). Takezaki *et al* (7) indicated that overexpression of GLI2DC, a C-terminal truncated form of GLI2, antagonized GLI transcription factor function, inhibited glioma-initiating cell proliferation in culture and neoplasms occurring in organisms; glioma-initiating cell proliferation was prevented by clipping glial downstream factor cell division cycle 2 (CDC2). These results suggested that the HH/GLI/CDC2 signaling cascade has an important role in glioma-initiating cell proliferation and malignancy. Since GLI2 affects downstream multiple carcinogenic and cancer-inhibiting pathways and is a key player in the network of neoplasmic microenvironments, the possibility of blocking multiple pathways by targeting GLI2 may be a promising strategy.

Expression and role of PTCH in GBM. PTCH is the receptor of HH protein. In vertebrates, two PTCH homologs have been isolated: PTCH1 and PTCH2 (82). PTCH1 is mainly expressed in SHH protein-producing mesenchymal cells, while PTCH2 is expressed in skin and testicular epithelial cells (83). A large clinical cohort study using the TCGA-GBM database detected GLI1 expression in relation to PTCH1. The strong correlation between GLI1 and PTCH1 expression was indicated to be a potential marker of HH-pathway activity (84), since PTCH1 is a true target of GLI1 transcription factors (85) and its expression is expected to increase with the activity of GLI1 (5). Marjanovic Vicentic *et al* (86) reported increased expression of HH ligand-receptor PTCH and HH effectors GLI1 and GLI2 in U87 and U251 cells overexpressing SOX3. BBF2H7 is an endoplasmic reticulum stranded transmembrane basic leucine zipper transcription factor that binds to HH ligand and PTCH1 to promote the formation of ligand-receptor complexes, thereby activating HH signal transduction (87). Iwamoto *et al* (88) further indicated that the c-terminal end of secreted lumen BBF2H7 participates in HH ligand-dependent GBM proliferation by binding to HH ligands and PTCH1 to activate HH signaling. Therefore, SOX3 and BBF2H7C terminals may become novel targets for anticancer drug development.

HH signaling pathway and the role of GSC in GBM. The HH, mTOR, Notch and Wnt/β-catenin signaling pathways are important signaling pathways that regulate GSC stemness and self-renewal (27,89,90), However, the self-renewal and abnormal differentiation of GSCs and their ability to promote the formation of drug resistance to RT and chemotherapy are the main reasons for the recurrence and invasion of GBM after conventional treatment (91,92). The mechanisms of how GSCs during invasion through the HH pathway, particularly in the face of complex and changing brain tissue anatomy, are presented in Fig. 2.

It has been reported that related homolog genes [e.g. Quaking homolog I)] (93), as well as transcription factors (e.g. Nanog homeobox) (94) and sialidase (e.g. neuroaminidase 4 (95) are able to activate the HH signaling pathway to maintain the self-renewal ability of GSCs by increasing SHH/GLI1 expression. This may promote the development of stem-like traits of GSCs and the formation and migration of GBM cell spheres (93-95). In addition, scaffold protein discs large homolog 5 (DLG5) and differentiation inhibitor 1 (ID1) regulate HH pathways by inhibiting downstream target ubiquitination (e.g. GLI1/2) and reducing GLI1/2 degradation (27,96). Cullin-3 interacts with GLI1/2 and dishevelled segment polarity protein 2 and induces their degradation through ubiquitination (27). ID1 and DLG5 inhibit cullin-3 ubiquitin ligase, activate HH signaling and promote GSC proliferation and tumorigenicity (27,96). Park *et al* (97) demonstrated that dihydro pyrimidine-associated protein 5 (DRP5) is particularly upregulated in the proneural (PN) subtype of GSC and has a key role in maintaining GSC characteristics, including tumor globule formation, stem cell marker expression and xenograft tumor growth, and DRP5 is considered to be a functional biomarker of GBM derived from PN-GSCs. The emergence and maintenance of CSCs are usually controlled by the tumor microenvironment. The tumor microenvironment always provides metabolic challenges to cancer cells and CSCs, mainly due to tissue hypoxia. Mondal *et al* (98) revealed that nutritional deprivation-induced enhanced the expression of specific biomarkers for GSCs, with higher invasiveness and angiogenic characteristics. These cells induced by microenvironmental nutritional stress (NS) have a high xenoefflux capacity and are therefore resistant to numerous anticancer drugs. The mechanism is that NS activates the Wnt and HH signaling pathways by regulating the β/AKT axis of β-catenin and GLI1, respectively. Vascular endothelial cells in the tumor microenvironment may provide SHH to further activate HH signaling pathways, thereby promoting GSC properties (99).

In summary, the neurobiology and basement membrane invasiveness of neural stem cells involves multiple molecular pathways that are interrelated. Therefore, targeting

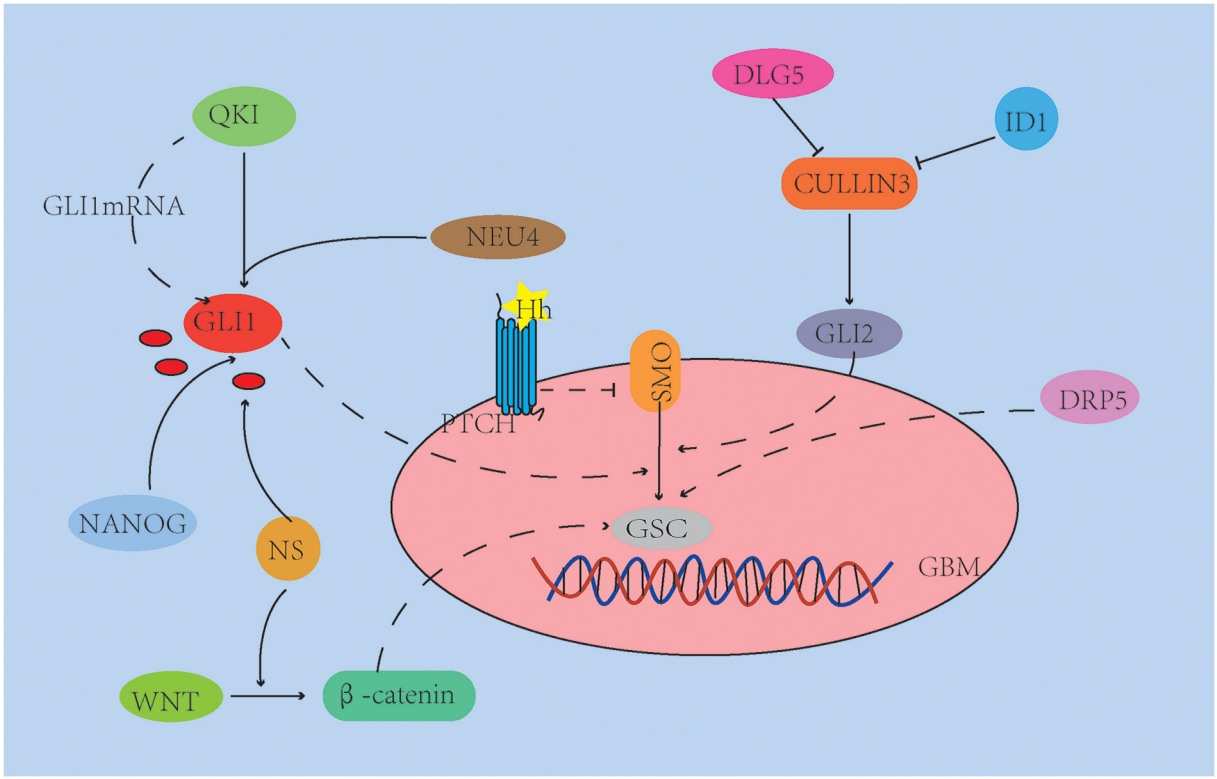

Figure 2. Related homolog genes (e.g. QKI), transcription factors (e.g. NANOG) and sialidase (e.g. NEU4) are able to activate the HH signaling pathway to maintain the self-renewal ability of GSCs by increasing SHH/GLI1 expression. ID1 and DLG5 inhibit cullin-3 ubiquitin ligase, activate HH signaling and promote GSC proliferation and tumorigenicity. DRP5 is specifically upregulated in the proneural subtype of GSC. NS activates the Wnt and HH signaling pathways by regulating the β/AKT axis of β-catenin and Gli1, respectively. Vascular endothelial cells in the tumor microenvironment may provide SHH to further activate HH signaling pathways, thereby promoting GSC properties. QKI, Quaking homolog; DRP5, dihydro pyrimidine-associated protein 5; SHH, Sonic Hedgehog; GBM, glioblastoma; SMO, smoothened; Gli, glioma-associated oncogene; GSC, glioblastoma stem cells; ID1, differentiation inhibitor 1; NS, microenvironmental nutritional stress; DLG5, discs large homolog 5.

cross-signaling pathways (e.g., Wnt/HH signaling) and specific markers may be a better therapeutic approach for GSCs.

5. Targeting the HH signaling pathway in GBM

The increased understanding of the key role of HH signaling in cancer has led to the development of pathway-specific inhibitors and the reuse of existing drugs that regulate HH/GLI (Table I). Drugs currently used in the clinic target SMO; among them, Vismodegib and Sonidegib have been approved by the US Food and Drug Administration for the treatment of basal cell carcinoma (BCC) (100,101) and medulloblastoma (101). However, mutations leading to drug resistance may occur, and thus, compounds that inhibit HH signaling downstream of SMO are urgently required and further research on the effects of HH/GLI pathway modulators in combination with anticancer drugs should be performed in order to provide evidence to pave the road for the future use of the combination of HH/GLI inhibitors and anticancer drugs.

Targeted therapy for GBM microenvironment. The mechanisms of GBM cell migration and invasion are complex and involve a series of mechanisms, including adhesion of GBM cells to the extracellular matrix (ECM) and ECM remodeling and degradation (102). As with other malignant tumor types, the growth, metastasis and invasion of GBM also depend on tumor angiogenesis. Although gliomas are characterized by hypervascularization, there are unavoidable disadvantages to anti-angiogenesis, such as reactive resistance mediated by the tumor microenvironment, and invasion and metastasis of tumor cells activated by hypoxia responses (103,104). During invasion and metastasis, GBM cells lose the polarized phenotype of epithelial cells and acquired mesenchymal characteristics, which is referred to as EMT (105). EMT is an active, drug-resistant, low-proliferative transient state that is frequently a feature of cancer as a whole but is seen in GBM in particular (106-108). Tubasatin A, a histone deacetylase 6 (HDAC6) inhibitor, reduced the expression of mesenchymal markers in GBM cells and contributed to the reversal of EMT (109). Feng *et al* (110) developed a pegylated poly (lactic acid) based nano-drug delivery system (nanoparticles) and modified CK peptides on its surface via GYG connectors to promote multitargeted delivery of Paclitaxel vasculogenic mimicry channels, tumor neovascularization and glioma cells. Similarly, Kast *et al* (111) proposed the EIS regimen (combination of itraconazole, metformin, naproxen, pirfenidone, quetiapine and rifampicin) that was able to safely and effectively block EMT of GBM. GBM progression may be inhibited by targeting tumor angiogenesis and EMT. Although these animal models are not perfect, they may be used to explore the effectiveness of new treatments for GBM prior to clinical phase I/II studies.

The HH signaling pathway is closely related to PC function, and thus, inhibiting PC function may help inhibit GBM proliferation, malignant development and treatment

Table I. List of hedgehog pathway inhibitors used in GBM.

Inhibitor name	Drug combination	Target	Mode of action	Reverse resistance	(Refs.)
Dynarrestin	(-)	PC	Inhibition of the flow of SMO in PC	(-)	(112)
O6-benzylamine	Honokiol(+)	SHH	Antagonist of MGMT	(+)	(117)
LDE225	(-)	SHH	Downregulated PTCH1 and GLI1	(-)	(120)
PEI-SNAs	(-)	GLI1	Binding to clearance receptors on GBM cells	(+)	(123)
GANT-61	TMZ	SHH	Increases production of ROS	(+)	(124)
GANT-61	(-)	SHH	Increases the expression of LC3 II and cleaved caspase 3 and 9	(-)	(125)
Curcumin	MicroRNA-326	SHH/GLI1	Antagonist of SHH/GLI1	(-)	(126)
XH30	(-)	GLI1	Decreases GLI1 activity	(+)	(127)
Phosphorylated peptides	(-)	GLI2	Decreases GLI2 activity	(-)	(128)
Tubasatin A	(-)	SHH/GLI1	Downregulation of GLI1 and PTCH1/2 receptors	(-)	(129,130)
CGP-2	(-)	GLI1	Antagonist of SMO	(-)	(132)
Capsulated	TMZ	GLI1	Inhibition of GLI1 expression	(+)	(136)
propylamine	(-)	SMO/GLI1	Antagonist of SMO/GLI1	(+)	(137)
PF403					

PC, primary cilia; TMZ, temozolomide; SMO, smoothened; MGMT, methylguanine methyltransferase; PEI-SNAs, polyethylene imine-coated spherical nucleic acid nanoparticles; ROS, reactive oxygen species; CGP-2, cyclodopamine glucuronoside precursor drugs; PF403, 13A (S)-3-hydroxyl-6,7-dimethoxyphenanthro[9,10-b]-indolizidine; Gli, glioma-associated oncogene; GBM, glioblastoma; SHH, Sonic Hedgehog; PTCH, patched; LC, light chain.

resistance (112). A previous study reported that the development of resistance to acquired kinase inhibitors is associated with upregulation of PC, uncontrolled PC length and abnormal activation of SHH signaling. Knockdown of KIF7 was observed to control the length and integrity of the PC and re-sensitize GBM cells (113). In addition, Dynarrestin was able to reversely inhibit intraflagellar transport of SMO flux in PC and inhibit HH pathway-dependent neuronal precursors and tumor cell proliferation (23). Therefore, Dynarrestin is a promising compound for the pharmacochemical development of anticancer drugs.

Inhibition of the HH/GLI pathway. HH signaling has been reported to be abnormally activated in >30% of solid tumor types, including GBM (62,114). Abnormal activation of the SHH pathway is associated with GBM resistance to temozolomide (TMZ) and the reason is the high expression of methylguanine methyltransferase (MGMT), which reverses the effects of TMZ on DNA (115,116) and confirms cell protection from TMZ-induced death by silencing three genes: MutS homolog 2 (a DNA repair protein involved in MMR), PTCH2 and chloride channel accessory 2 (a type 1 transmembrane protein that inhibits the Wnt pathway) (24). Resistance to TMZ was only slightly reversed by MGMT inhibitor O6-benzylamine, but a marked further enhancement was achieved by addition of Honokiol (117). Furthermore, the invasion of GBM was reported to be associated with the presence of CSCs and the SHH pathway has an important role in the maintenance and proliferation of CSCs (118,119). After inhibiting SHH, LDE225 slowed down the growth of GBM and downregulated PTCH1 and GLI1 *in vivo* (120). CSCs preferentially activate the DNA damage checkpoint response and exhibit enhanced DNA repair ability; thus, SHH signaling via GLI1 in CSC has a role in GBM resistance to TMZ (121).

Glabrescione B is the first small molecule to bind to GLI1 zinc fingers, impelling GLI1 activity by interfering with its interaction with DNA. Thus, it inhibits the ability of HH-dependent tumor stem cells to self-renew and cladogenesis. The determination of the structural requirements for GLI1/DNA interactions highlights their relevance to drug interference with GLI signaling (10). Melamed *et al* (122) developed polyethylene imine-coated spherical nucleic acid nanoparticles (PEI-SNAs) targeting GLI1. GLI1 PEI-SNAs bind scavenger receptors on GBM cells and undergo endocytosis in a pit/lipid raft/dynein-dependent manner, promoting the silencing of HH pathway genes and downstream target genes. These genes promote an aggressive, drug-resistant GBM phenotype. GLI1 PEI-SNAs not only significantly increased the sensitivity of nerve spheres to chemotherapy, but also further impaired the formation of dry nerve spheres (123).

Arsenic trioxide also significantly reduced the cladogenesis of tumor neuroglobules by inhibiting the HH pathway, inhibiting the proliferation of GBM neuroglobules and promoting apoptosis (124). The combination of the SHH inhibitor GANT-61 with TMZ increased the cytotoxicic effect of TMZ and the combination of GANT-61 with TMZ increased the production of reactive oxygen species in GBM cells, suggesting that inhibition of the SHH pathway may sensitize GBM cells to the effects of TMZ by increasing oxidative stress (114,124). GANT-61 induced apoptosis and autophagy in GBM cells by increasing the expression of light chain 3II and lysed Caspase-3 and -9 (125). Furthermore, GLI inhibition combined with TMZ increased the apoptosis rate of glioma stem cells by 6.8-fold, thereby reducing the size and number of nerve spheres grown from glioma stem cells (115). Yin *et al* (126) reported that the combination of tumor suppressor gene miR-326 and curcumin significantly inhibited the SHH/GLI1 pathway of glioma cells, independent of the P53 status, significantly increased apoptosis and reduced the proliferation and migration of glioma cells. Similarly, Ji *et al* (127) reported that a novel PI3K inhibitor, XH30, inhibited tumor growth that was resistant to TMZ. In terms of the mechanism, the role of XH30 may be to reverse the activation of GLI1 induced by SHH by atypical HH signaling and to reduce GLI1 activation by insulin-like growth factor 1 (127). Thus, XH30 may be a novel treatment option for TMZ-resistant GBM.

Traditional treatments for GBM include systemic chemotherapy, RT and surgery. Han *et al* (128) synthesized three phosphorylated peptides derived from GLI2 and combined them with the cell-penetrating peptide TAT-[47-57] AYGRKKRRQRRR. The three mixed phosphorylated polypeptides derived from GLI2 significantly increased the level of GLI2 phosphorylation and decreased the transcriptional activation of GLI2, and the radiation sensitization of GBM cells was significantly higher than that in the control group (128). HDAC6 was upregulated in GSCs and inhibited HDAC6 down-regulated glioma-associated oncogene GLI1 and PTCH1/2 receptors, as well as SHH signaling components, expression and activity, thereby inhibiting GSC proliferation, inducing differentiation and increasing the apoptosis rate through the SHH/GLI1 signaling pathway (109,129). Inhibition of HDAC6 by Tubasatin A enhanced the radiosensitivity of GBM tumor cells. The mechanism may be that HDAC6 inhibits checkpoint kinase (CHK)1 degradation induced by down-regulation of X-linked inhibitor of apoptosis, which reduced the DNA damage repair ability of GSCs, leading to increased radiosensitivity (109,130).

In summary, target genes associated with the SHH/GLI pathway provide promising new drug targets for inhibiting GBM proliferation, as well as overcoming drug resistance and radiation resistance of GSCs.

SMO inhibitors. The steroidal alkaloid cyclopamine, an antagonist of the HH coreceptor SMO, acts as an inhibitor of the HH pathway (131). To limit the toxicity of cyclodopamine to HH-dependent non-tumor cells, cyclodopamine precursor drugs [e.g., cyclodopamine glucuronoside precursor drugs (CGP-2) and 1b] are commonly used (132,133). It was indicated that CGP-2 inhibits the HH pathway more effectively than conventional TMZ adjuvants (131). In the presence of β-glucuronidase, the activated prodrug 1b was toxic and downregulated the HH target gene GLI1 in C6 cells and C6-CSCs (132). In U251 cells, tyramine not only inhibited the HH/GLI1 signal transduction pathway, leading to decreased MGMT expression, but also induced cell apoptosis by activating caspase-3 cleavage, thus leading to increased sensitivity of GBM to TMZ (133). However, the combination of acepromazine and TMZ enhanced the dryness and drug resistance of GBM cells by inducing the expression of SOX-2 and OCT-4 and may lead to tumor recurrence in patients (134). Therefore, the best therapeutic strategy is to first inhibit the SHH pathway and then administer TMZ (134,135). Liu *et al* (136) found that the combination of capsulated propylamine and TMZ had synergistic cytotoxic effects and was more likely to inhibit the ability to induce apoptosis and eliminate neuroglobin formation by inhibiting GLI1 expression. Therefore, MCyp may be used as a tumor stem cell inhibitor to prevent tumor recurrence. Future efforts should be made to investigate the possibility of using HH pathway inhibitors prior to conventional chemotherapy in patients with GBM. Future efforts should focus on the efficacy of HH pathway inhibitors prior to systemic chemotherapy in patients with GBM.

Chen *et al* (137) indicated that PF403 inhibits cell surface Smoothened (Smo) receptor aggregation at the molecular level by directly binding or enhancing the interaction between Smo and the suppressor PTCH1. In addition, PF403 significantly inhibited the transcription of GLI1 and its accumulation in the nucleus by promoting the interaction between SUFU-GLI1 and PKA-GLI1, blocking the HH signaling pathway of T98G MGMT-expressing cells, and downregulated the expression of MGMT. Inhibition of the HH pathway by PF403 counteracted TMZ resistance and the precursor Cat3 of PF403 enhanced the anti-tumor activity of TMZ *in vivo* (137,138). In summary, Cat3 is a promising therapeutic agent for HH-driven GBM.

6. HH pathway and immunotherapy

The key to antitumor immunity is that antigen-presenting cells (APCs) engulf tumor cells. TMZ may induce an endoplasmic reticulum stress response, and the combination of CD47 blocker and TMZ may produce significant prophagocytosis (139,140). Increased tumor cell phagocytosis, enhanced antigenic cross-presentation in APC and activation of cyclic GMP-AMP interferon gene synthase stimulation leads to more efficient T-cell effects. This connection between innate and adaptive responses inhibits GBM growth while also activating immune checkpoints. Sequential administration of an anti-programmed cell death protein 1 (anti-PD1) antibody overcomes this potential adaptive resistance (140). However, the mechanism by which anti-PD1 antibodies reverse GBM resistance through HH signaling remains to be elucidated. It has been reported that GANT-61 is able to reduce the expression of PD-L1 and the proliferation of tumor cells *in vivo* and *in vitro* by using organic compound drugs for human gastric cancer. Of note, anti-PD-L1 antibodies induced apoptosis of tumor cells in organs of GLI2-expressing mice. Studies suggested that GLI2 expressed in gastric cancer cells is an internal regulator of PD-L1 and promotes tumor growth by inhibiting the anti-tumor response (141,142). In summary, the HH pathway may become a new immunotherapy target for GBM after further study.

7. Discussion

The biological treatment of GBM has been studied for numerous years, but the treatment of deadly cancers still poses a great challenge. GBMs are highly invasive and susceptible to drug resistance, resulting in a high mortality rate, and GBM accounts for 2.9% of cancer-related deaths (143).

A key treatment issue for GBM is the high degree of heterogeneity within the tumor. This heterogeneity further complicates the differences among patients with GBM. In addition to heterogeneity, GBM also has GSCs that contribute to tumor proliferation, maintenance and drug resistance (144,145), and GSCs may respond differently to TMZ or ionizing radiation (146). All of this makes routine treatment difficult. Further research is required on the impact of GBM heterogeneity on modern therapies, including molecular immunotherapy and personalized therapy. The lymphocytes present in GBM have an increased proportion of CD4+T cells and FOXP3+ regulatory T cells may induce signaling pathways that inhibit immune responses (147,148), e.g., the expression of IDO enzyme and STAT3 signals (149,150). However, GBM tumor-infiltrating effector lymphocytes were observed to be rare (151,152). This may also be the reason why a clinical trial of immune checkpoint blocking using the anti-PD1 antibody nivolumab (NCT02017717) used in patients with newly diagnosed or relapsed unmethylated GBM (153), have not been successful. The currently used immunotherapy for GBM may be broadly divided into vaccine therapy, immune checkpoint blocking, oncolytic virus therapy and chimeric antigen receptor T-cell therapy (154-156). In addition to immunotherapy, EGFR using tyrosine kinase inhibitors (TKI), VEGF TKI and targeted therapies for the PI3K/mTOR pathway have also been explored in GBM. However, a phase 3 trial of deatuxizumab mandolin in combination with standard therapy for the treatment of newly diagnosed EGFR-amplified GBM was terminated early for being ineffective (157), and mTOR inhibitors such as everolimus (NCCTGN057K) and Taxiolimus (EORTC26082) also proved to lack efficacy in phase 2 trials (158,159). A phase 2 trial of regorafenib (REGOMA) in a relapsed setting indicated a therapeutic OS benefit compared to lomustine, but the drug had minimal activity; thus, VEGF monotherapy may have a limited effect in a non-selected population (160).

It is necessary to study new targets for the treatment of GBM. HH signaling has emerged as an attractive target for cancer therapy and several HH inhibitors have been designed. To date, SMO inhibitors were proven to have satisfactory efficacy in BCC and medulloblastoma (100,101), but clinical trials for other cancer types, such as colorectal, pancreatic or lung cancers, have yielded poor results (161-163). In preclinical studies, compared with HH and SMO inhibitors, GLI inhibitors had better anticancer efficacy (164,165). In addition, GLI inhibitors effectively inhibited the growth of numerous GLI-dependent cancers by targeting the GLI-regulated SMO-independent pathway (166). As for inhibitors of GLI1 and GLI2 transcription factors, the anticancer drug arsenic trioxide is currently the only drug undergoing clinical trials in solid tumors and hematological malignancies (167). Although the use of HH inhibitors in GBM has not been extensively investigated, numerous studies suggested that HH inhibitors in combination with conventional therapies may markedly increase efficacy and reduce the incidence of drug resistance (124-126,133,137). Of course, this also requires a large number of clinical trials to further verify whether HH inhibitors are beneficial to the therapeutic efficacy of GBM.

Epigenetic regulators interact with drivers of GBM stem cell-like cell proliferation. These drivers include Notch, HH and WNT pathways. Previous studies suggested that these signaling pathways may perform cross-talk with SHH signaling pathways (27,71,80-81,89,90), which means that these signaling pathways may be activated simultaneously in different tumor types. WNT/β-catenin interacts with the SHH pathway through GLI1 and GLI2 by regulating the expression of secreted crimp-related proteins. SHH signaling was inhibited by GSK3β, a component of the WNT signaling pathway. In certain tumor types, upregulation of the WNT signaling pathway occurs sequentially when the SHH pathway is inhibited (168). In addition, the synergistic effect of the inhibition of the SHH and PI3K/AKT/mTOR signaling pathways may inhibit the proliferation of glioblastoma-initiating cells (GICs), tumor growth and the formation of neural spheres and clones, and induce cell apoptosis (169). Combined drug action targeting two pathways or inhibition at the intersection of two pathways may be a good breakthrough point for targeted therapy.

8. Conclusion

Current conventional therapies for GBM are ineffective due to drug resistance issues and resistance may be overcome through a combination of HH inhibitors or multilevel HH signaling cascades, such as combinations of multiple targeted HH drugs and multi-target HH inhibitors. In addition to pioneering new approaches based on existing scientific theories, the effectiveness of evaluating these therapies in clinical trials requires to be further improved. This includes increasing the number of patients with GBM in phase I trials of HH pathway inhibitors, thereby providing more complete clinical trial data for the development of more effective targeted therapeutic strategies.

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Authors' contributions

HW performed the literature search and selection. XX was responsible for the conception, analysis and design of the study. HW and XX were major contributors in writing of the manuscript. DW and JP participated in the coordination of the study and reviewed the manuscript. BT and YG were responsible for the revision of the manuscript. QL and ZG were responsible

for the literature search and design of the study. All authors read and approved the final manuscript. Data authentication is not applicable.

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Competing interests

The authors declare that they have no competing interests.

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