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### ► **To cite this version:**

Florence Lefranc, Emilie Le Rhun, Robert Kiss, Michael Weller. Glioblastoma quo vadis: Will migration and invasiveness reemerge as therapeutic targets?. *Cancer Treatment Reviews*, 2018, 68, pp.145-154. 10.1016/j.ctrv.2018.06.017 . inserm-02940699

**HAL Id: inserm-02940699**

**<https://inserm.hal.science/inserm-02940699>**

Submitted on 16 Sep 2020

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## Hot Topic

## Glioblastoma *quo vadis*: Will migration and invasiveness reemerge as therapeutic targets?



Florence Lefranc<sup>a,\*</sup>, Emilie Le Rhun<sup>b,c,d</sup>, Robert Kiss<sup>e</sup>, Michael Weller<sup>f</sup>

<sup>a</sup> Department of Neurosurgery, Hôpital Erasme; Université Libre de Bruxelles, Route de Lennik 808, 1070 Brussels, Belgium

<sup>b</sup> University of Lille, U-1192, F-59000 Lille, France

<sup>c</sup> Inserm, U-1192, F-59000 Lille, France

<sup>d</sup> CHU Lille, General and Stereotaxic Neurosurgery Service, F-59000 Lille, France

<sup>e</sup> FRS-FNRS, Brussels, Belgium

<sup>f</sup> Department of Neurology, University Hospital & University of Zurich, Frauenklinikstrasse 26, CH-8091 Zurich, Switzerland

## A B S T R A C T

**Purpose:** The purpose of the current review is to highlight, on one hand, the fact that the migratory pattern of glioma cells is the major obstacle to combat them with chemotherapy, and on the other one, the new treatment strategies to overcome this obstacle.

**Methods:** This review surveys several membrane and extracellular molecules involved in glioma cell migration, invasiveness and resistance to apoptosis.

**Results:** This review focuses on signaling pathways implicated in the positive regulation of glioblastoma cell migration, including glutamate and ion channel networks, microtubules and membrane-derived extracellular vesicles (EV) containing microRNAs. Glioma cells release glutamate to the extracellular matrix, inducing neuronal cell death, which may facilitate glioma growth and invasion. Glioma cell migration and invasion are further facilitated through ion channels and transporters that modify cellular volume. Microtubules and EV promote connections and communication among glioma cells and with the microenvironment and are associated with progression and resistance to therapy. Potential therapies linked to these pathways for glioblastoma are being developed.

**Conclusion:** Our view is evolving from an intracellular view of the complex intracellular signaling pathways to one of orchestral machinery, including connections between heterogeneous tumoral and nontumoral cells and with the microenvironment through channels, microtubules, and extracellular miRNA, generating different signals at different times. All of these elements give rise to a new perspective for the treatment of glioblastoma.

### Introduction

Gliomas, including the most common variant, glioblastoma, are the most common primary malignant brain tumors, with an annual incidence of 5.26 cases per 100,000 population or 17,000 new diagnoses per year [1]. Glioblastoma remains one of the most lethal forms of cancer. The high spatial and temporal glioblastoma heterogeneity due to functionally diverse cell types, chaotic leaky vasculature and tumor cell infiltration contributes to poor prognosis in patients [2]. The current understanding of the biology and genetics of glioblastoma has greatly improved in the past two decades. Two major multi-institutional programs, the Glioma Molecular Diagnostic Initiative and The Cancer Genome Atlas (TCGA), have pursued comprehensive genomic characterization of a large number of clinical glioma samples using a variety

of technologies to measure gene expression, chromosomal copy number alterations, somatic and germline mutations, DNA methylation, microRNA, and proteomic changes. These data will facilitate the discovery of therapeutic and diagnostic targets and pathway candidates, validation of research and clinical observations and generation of unanticipated hypotheses that can advance the molecular understanding of this lethal cancer [3]. Sturm et al. identified six epigenetic glioblastoma subgroups displaying characteristic global DNA methylation patterns, harboring distinct hotspot mutations, DNA copy-number alterations, and transcriptomic patterns [4]. Brenann et al. provided a comprehensive and in-depth overview of the genetic, epigenetic and transcriptional profiles associated with glioblastoma [5]. The new WHO 2016 classification, therefore, is not only based on the histological features of the tumor but also includes isocitrate dehydrogenase (IDH)

\* Corresponding author at: Service de Neurochirurgie – Hôpital Erasme- Université Libre de Bruxelles, Route de Lennik, 808, 1070 Brussels, Belgium.  
E-mail address: [Florence.lefranc@erasme.ulb.ac.be](mailto:Florence.lefranc@erasme.ulb.ac.be) (F. Lefranc).

<https://doi.org/10.1016/j.ctrv.2018.06.017>

Received 14 January 2018; Received in revised form 19 June 2018; Accepted 21 June 2018

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mutation and chromosomal arms 1p and 19q codeletion status to classify diffuse gliomas of adulthood [6]. However, each individual glioblastoma should be considered as an ensemble of molecularly distinct subclones that reflect a spectrum of dynamic cell states. It is becoming clear that glioblastoma is a composite of multiple cell populations in the same tumor, and this heterogeneity makes the design of effective treatment modalities challenging [7,8].

#### *Diffuse glioma genesis*

In the context of gliomagenesis, glial cells were once considered the only dividing cells in the postnatal brain, making these cells the only brain cells susceptible to transformation [9,10]. It is now known that both multipotent and self-renewing neural stem cells and self-renewing glial progenitor cells produce astrocytes and oligodendrocytes in limited regions of the adult brain including the subventricular zone, the subgranular zone of the dentate gyrus and possibly other minor stem-cell niches throughout the brain [11–13]. These stem cell and progenitor elements, in addition to differentiating into adult glia, are now thought to constitute a substrate for neoplastic transformation, termed glioma initiating cells or glioma stem cells (GSCs), a small population of self-renewal, therapy-resistant and slow-dividing malignant cells inside the main tumor bulk. Calabrese et al. found that a significant proportion of vessel-associated Nestin + tumor cells are proliferating and that these cells are distributed throughout tumors, from all regions of the cerebrum and cerebellum [14]. In the early 70 s, Pierce proposed that tumors arise from carcinogenic events occurring in stem cells of a particular tissue that cause a defect in the control of normal stem cell function, including self-renewal and differentiation [15]. In the adult brain, neural stem cells commonly possess features associated with gliomas, including a robust proliferative potential, high motility, diverse progeny, associations with blood vessels and white-matter tracts and an immature gene expression profile characterized by expression of nestin, epidermal growth factor receptor (EGFR), Notch and PTEN, telomerase activity, and Hedgehog and Wnt pathway activities [16–19].

GSCs express nestin (an intermediate filament protein) and CD133 (also known as prominin 1, a transmembrane glycoprotein), factors associated with neural stem cells, although some GSCs are CD133-negative [20]. Although CD133 expression seems to be related to stemness, it might only indicate an intermediate, adaptive state of a cell rather than a stable phenotype [21]. A tumor is therefore a caricature of normal tissue and appears to be undifferentiated because of the preponderance of undifferentiated proliferating stem cells in relationship to the number of cells that have differentiated and are post-mitotic.

Stem and progenitor cells in the central nervous system may be particularly at risk for malignant transformation since they constitutively activate the cellular machinery necessary for tumor initiation, progression or both (including growth factor receptor EGFR, PI3K, Akt, mTOR, Ras, mitogen-activated protein kinase (MAPK) members). Thus, neural stem cells may represent the path of least resistance in tumorigenesis since these cells already have the ability to bypass apoptosis and senescence. As a result, neural stem cells may require less than the estimated four to seven mutations needed to induce malignant change in a differentiated cell [22]. In gliomas, as in several solid tumors outside the central nervous system, only a small fraction of the total population of cancer cells are clonogenic cancerous neural stem cells. However, this resistant population is considered the primary cause of glioblastoma invasion and responsible for the maintenance of a minimal residual disease, which commonly leads to post-therapeutic recurrence [23]. Evidence from human gliomas and rodent glioma models indicate that the glioma vascular niche supports glioma stem cell maintenance and nurtures tumor growth by offering nutrients necessary for unlimited glioma stem cell proliferation [14,24]. Several embryonic signaling pathways, such as Notch, Hedgehog and Wnt/b-catenin, have been reported to help maintain the GSC pool and thus

provide potentials target for treating glioblastoma [25]. Paw et al. [26] and Lee et al. [27] recently provided an overview of the specific signaling pathways that regulate glioblastoma stem cell maintenance and migration.

On the basis of their histological appearance, gliomas have been traditionally classified as astrocytic, oligodendroglial or ependymal tumors and assigned WHO grades I-IV, which indicate different degrees of malignancy. Diffusely infiltrating gliomas in adults are now separated into three overarching tumor groups with distinct natural histories, responses to treatment and outcomes: IDH-mutant, 1p/19q codeleted tumors with mostly oligodendroglial morphology that are associated with the best prognosis; IDH-mutant, 1p/19q non-co-deleted tumors with mostly astrocytic histology that are associated with intermediate outcome; and IDH wild-type, mostly higher WHO grade (III or IV) tumors that are associated with poor prognosis [28].

#### *Treatment*

The current standard treatment for glioblastoma includes surgical resection, followed by radiotherapy plus concomitant temozolomide (TMZ) followed by six cycles of maintenance TMZ [29,30]. These treatments allow a median overall survival in the range of 16 months in clinical trial patient populations, but are never curative. Indeed, less than 10% of the 77,000 patients diagnosed each year with glioblastoma in the USA and Europe will survive more than 5 years. This dismal prognosis reflects the high rate of tumor recurrence approaching 100% which is linked to the profoundly infiltrative nature of this tumor, rendering curative surgical resection elusive, and its relative resistance not only to the conventional treatments of genotoxic radiotherapy and chemotherapy, but also more recently to targeted therapies. Anti-angiogenic therapies have dominated the clinical trial landscape of glioblastoma. However, it has now become clear that this approach is hopeless, at least with a view to prolonging overall survival. Indeed, while anti-angiogenic treatment targeting VEGF exerts anti-edema effects in glioblastoma which may translate into clinical benefit in subgroups of patients, no clinical trial has demonstrated a survival benefit afforded by anti-angiogenic agents.

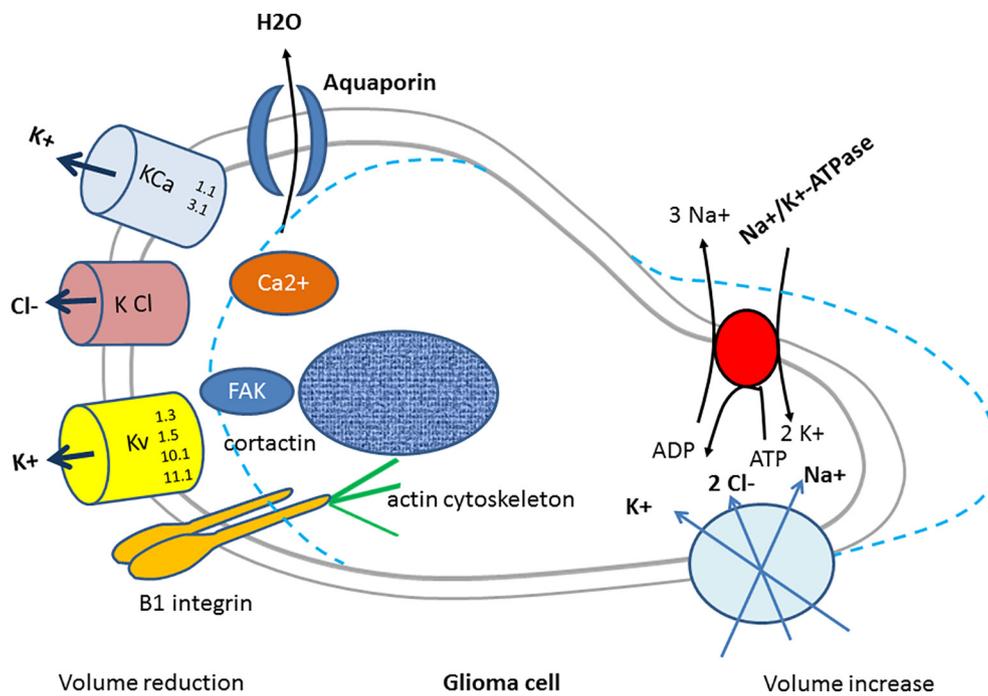
#### **Glioblastoma migration: What is new?**

In a previous review we highlighted the complex dynamic processes governing glioma cell migration in general and in glioblastoma in particular [31]. This dynamic process includes, among others, (i) cell adhesion to numerous components of a modified (when compared to normal parenchyma) extracellular matrix (ECM); (ii) cell motility, which involves the reorganization of the actin cytoskeleton, primarily through modifications in the field of integrin network–ECM component interactions; and (iii) invasion, which involves the degradation of matrix proteins by tumor-secreted proteolytic enzymes, primarily serine proteases, cathepsins and metalloproteinases (MMPs). Admittedly, no effective treatments for human glioma patients have evolved from these avenues of research.

In the present review, we analyzed the recent literature on new promising therapies to combat glioblastoma with a focus on two pathognomonic features of glioblastoma biology, i.e., glioblastoma cell migration and invasion through the brain parenchyma and, associated with that, the apparent intrinsic resistance of migrating glioblastoma cells to pro-apoptotic stimuli, including cytotoxic chemotherapy and radiotherapy.

We focused in the current review on glutamate, ion channels, tumor microtubules and several signaling pathways. While there is a myriad of signaling pathways that influence the migratory behavior of malignant astrocytes [31], we decided to restrain ourselves on the “glutamate pathway” because we are convinced that impairing this malignant astrocyte-migration avenue of invasion within the brain parenchyma would actually contribute therapeutic benefits to the patients. We detail





podium for leading edge extension.

#### Potassium channels

Among ion transporters, potassium channels show the highest variability and the most frequently altered pattern of expression in many tumor types [49]. Cell cycle, proliferation, migration, invasion, angiogenesis and apoptosis are all processes that can be modified by the expression of potassium channels [50]. Potassium channels have been implicated in every step of adhesion and migration [45]. Interactions of potassium channels with crucial contributors to cell adhesion, such as FAK and cortactin, an actin-interacting protein implicated in the cytoskeletal architecture and often amplified in several types of cancer, have been reported (Fig. 2) [51]. Potassium channels also associate with the main contributors to cell adhesion signaling, integrins. The most important understood mechanisms by which particular potassium channels contribute to cancer initiation and progression have recently been reviewed [48]. Here we focus on the literature on potassium channels involved in glioma progression and migration, e.g. Kv1.3 and Kv1.5 [52–54], Kv10.1 [55–57], Kv11.1 [58], KCa1.1 [59,60] and KCa3.1 [61] (Fig. 2).

The Kv1.3, Kv10.1, and Kv11.1 channels are the best studied voltage-gated K channels in the context of cell migration. They are of particular importance for cells of the immune system and tumor cells, including glioblastoma cells, constituting potential diagnostic or prognostic markers or even therapeutic targets [48,50].

Kv10.1 (also known as EAG1, ether-à-go-go and KCNH1), a voltage-gated potassium channel that is virtually absent from normal cells outside the central nervous system, is expressed in limited areas of the brain (hypothalamus, hippocampus, cerebral cortex, cerebellum and olfactory nerve) but highly expressed in different cancers, including brain metastases and gliomas [57]. Bai et al. also observed Kv10.1 overexpression in both glioma cell lines and clinical glioma samples [55]. In several different cancers, Kv10.1 enhances proliferation. It has also been shown to be required for the maintenance of growth associated with the p53/miRNA34/E2F1 regulatory pathway in SHSY5Y human dopaminergic neuroblastoma cells [62]; this channel up-regulates HIF-1 and VEGF in the tumor environment [63] and increases migration [64]. Kv10.1 expression in Chinese Hamster Ovary (CHO) cells affects cytoskeletal organization, which might influence cancer cell proliferation, migration and metastasis [64].

**Fig. 2.** Glioma cell volume and shape regulation: involvement of ion channels. Cell migration and glioblastoma cell migration in particular is characterized by the polarized cellular and molecular processes that promote the protrusion of the leading edge (local volume increase) and retraction of the trailing edge (local volume reduction). A net outflow of ions and water at the cell trailing edge and a net inflow of ions and water at the cell leading edge underlie these localized hydrodynamic changes. Ion channels and transporters display polarized subcellular distribution. At the trailing edge, calcium entry through calcium-permeable channels can activate calcium-activated potassium channels (KCa) to promote potassium efflux. The opening of chloride (KCl) and potassium channels (Kv) further enhances the outflow of ions  $\text{Cl}^-$  and  $\text{K}^+$ , followed by obligatory water efflux through aquaporins. At the leading edge, an assortment of inward ion channels, such as the  $\text{Na}^+/\text{K}^+$  ATPase and the  $\text{Na}^+-\text{K}^+-\text{Cl}^-$  cotransporters (NKCC1) together with aquaporins, regulate ion and water influx. This ion and water influx drives local cell volume increase in the protruding lamelli-

A correlation between dismal prognosis and Kv10.1 expression in patients with brain metastasis from different carcinomas has been observed, and metastatic cancer patients receiving tricyclic anti-depressants (such as imipramine, chlorimipramine, citalopram and amitriptyline) showed a significantly longer median overall survival than patients not treated with these agents [57].

Kv11.1 (also known as HERG or KCNH2) was among the first voltage-gated channels directly associated with cancer [65]. Further, Kv11.1 blockers impair the proliferation of tumor cells [66]. Kv11.1 physically interacts with  $\beta 1$  integrin at the plasma membrane of human cancer cells to regulate survival and migration [67] (Fig. 2), and this channel has also been implicated in the regulation of apoptosis of human glioblastoma cells (see below) [68].

The Kv1.3 and Kv1.5 channels modulate  $\text{Ca}^{2+}$  signaling and cell volume and have been analyzed in a number of cancer cell types [69]. The levels of expression of the Kv1.5 and Kv1.3 channel subtypes vary between different glioma groups, and differential expression of Kv1.5 according to malignancy grade has been noted [52]. Kv1.5 also contributes to the resistance to pro-apoptotic stimuli of cancer cells, including gliomas (see below).

The calcium-activated potassium channels KCa1.1 and KCa3.1 are important for the migration of glioma and glioma stem cells [61,70,71]. The REpository of Molecular BRAIn Neoplasia DaTa (REMBRANDT) database allowed to determine that the gene KCNN4, which encodes KCa3.1, is overexpressed in 32% of gliomas, and that its expression correlated with short survival [70]. Turner et al. generated U251 glioma cells that stably express an inducible knockdown shRNA to experimentally eliminate KCa3.1 expression [70]. Subjecting these cells to a combination of *in vitro* and *in situ* invasion assays revealed that KCa3.1 expression may enhance glioma invasion and either specific pharmacological inhibition with TRAM-34 or elimination of this channel by gene silencing impairs invasion. Importantly, after intracranial implantation into SCID mice, ablation of KCa3.1 with inducible shRNA from U251 cells resulted in a significant reduction in tumor invasion into the surrounding brain [70].

Migration of glioblastoma cells can be stimulated by ionizing radiation (IR) [72]. Steinle et al. accordingly analyzed the IR-induced increase in  $\text{Ca}^{2+}$ -activated potassium channel activity and its effect on

the migration of T98G and U87MG cells [73]. IR dose-dependently stimulated migration of glioblastoma cells, which in turn was sensitive to the channel inhibitor paxilline. These authors concluded that IR stimulates potassium channel activity, resulting in the activation of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II and enhanced glioblastoma cell migration [73].

#### Chloride channels

Approximately 20 years ago, Sontheimer proposed that the invasive migration of human glioma cells into the normal brain parenchyma requires shape and/or volume changes [74]. Chloride currents may contribute to such shape-volume changes by affecting net salt fluxes across glioma cell membranes, with accompanying water efflux, resulting in cell shrinkage that is conducive to glioma cell migration through minute extracellular spaces of the brain [75]. Sontheimer and collaborators subsequently performed a direct examination of  $\text{Cl}^-$  dynamics in human glioma cells and its functional relationship to cell biology [76]. First, these authors observed that invasive tumor cells become morphologically polarized and develop membrane protrusions (Fig. 2). During this process, invasive glioma cells alter their cell shape and volume to fit into the narrow extracellular spaces available and invade the brain parenchyma. These features include secretion of  $\text{Cl}^-$  and  $\text{K}^+$  through ion channels localized to lipid raft domains on invadopodia and water passively flowing through water channels or aquaporins [46]. In addition, the interplay between  $\text{Na}^+$ - $\text{K}^+$ - $\text{Cl}^-$  co-transporters and  $\text{Na}^+$ / $\text{K}^+$  ATPase leads to active accumulation of  $\text{K}^+$  and  $\text{Cl}^-$ , establishing a gradient for  $\text{KCl}$  efflux (Fig. 2) [46].

Here, we do not review the roles of other channels involved in glioma migration, such as transient receptor potential (TRP) calcium channels, which also contribute to changes in  $\text{Ca}^{2+}$  by modulating the driving force for  $\text{Ca}^{2+}$  entry as Morrone et al. recently summarized this subject [77].

#### Microtubules

Osswald et al. reported that astrocytomas but not oligodendrogliomas develop functional multicellular network structures through ultra-long membrane protrusions and that astrocytoma cells use these distinct tumor microtubules as routes for brain invasion, proliferation, and interconnections over long distances [78]. The resulting network facilitates multicellular communication through microtubule-associated gap junctions. Confocal microscopy revealed punctate connexin 43 (Cx43) gap junction immunoreactivity, particularly at the tumor microtubules of astrocytoma cells, which was not observed for other connexins. When damage to the network occurs, tumor microtubule is used for repair. Moreover, microtubule-connected astrocytoma cells, but not those remaining unconnected throughout tumor progression, were protected from cell death inflicted by radiotherapy. Osswald et al. hypothesized that intercellular tumor microtubules can serve as an individual tumor cell to distribute critical elevations of small molecules, such as calcium, within the large network (Fig. 3), achieving nonlethal levels for radiotherapy-induced cytotoxicity and for intrinsic apoptotic cell death in glioma cells [78]. Neuronal growth-associated protein 43 (GAP-43) is important for microtubule formation and function and drives microtubule dependent tumor cell invasion, proliferation, interconnection, and radioresistance. The disconnection of astrocytoma networks by disrupting their tumor microtubules may emerge as a new principle to reduce the treatment resistance of this disease, but clinical applications have not been introduced [78].

#### Extracellular MicroRNAs

Over the past decade, there has been increased awareness of the possible role of microRNAs (miRNAs) in the development of cancer, including glioblastoma [79]. miRNAs are small non-coding RNAs of approximately 20–23 nucleotides that act as post-transcriptional

regulators of gene expression. miRNAs repress gene expression by binding to complementary sequences in the 3' untranslated region (3' UTR) of mRNAs and targeting these molecules for degradation, thereby preventing their translation, resulting in a decreased level of encoded proteins that affect an array of cellular processes, e.g., differentiation, proliferation, migration, metabolism, apoptosis and stem cell maintenance [80]. Aberrant miRNA biogenesis in cancer occurs at different steps during miRNA maturation [79], and a single miRNA can regulate the expression of many genes. Depending on its target genes, miRNA can act as a tumor suppressor or tumor promoter. The accumulation of specific miRNAs in glioblastoma samples from patients and in glioblastoma cell lines was initially demonstrated a decade ago [81,82]. Since then, numerous reviews have highlighted the involvement of miRNAs in glioblastoma progression, migration and radio-chemotherapy resistance and suggested their potential as therapeutic targets [83–89].

Recently, microRNAs have also been localized to the extracellular space, often encapsulated in secreted extracellular vesicles (EV), nanometer size membrane-enclosed particles formed either from the fusion of an endosome with the plasma membrane (exosomes) or directly from the cell membrane (microvesicles) and released from numerous cell types, including glioblastoma cells, to mold the tumor microenvironment to their advantage (Fig. 3) [90–92]. Neighboring or distant recipient normal and tumor cells can take up EVs containing tissue-specific expressed/secreted microRNAs [93]. In a recent review, Rooj et al. discussed the current knowledge regarding the multifaceted roles of both cellular and EV microRNAs in shaping the glioblastoma microenvironment [92]. Glioblastoma EVs are enriched in several oncogenic microRNAs, including miR-21, miR-23a, miR-30a, miR-221 and miR-451 [94]. However, the functions of most of these microRNAs remain unknown. Hoshino and colleagues elegantly demonstrated that tumor-derived EVs are internalized in organ-specific cells, and this process plays an important role in the preparation of the pre-metastatic niche [95]. EVs containing miRNA were also detected in the blood or the cerebrospinal fluid of glioblastoma patients [96]. Thus, microRNAs circulating in complexes with tumor-originated EVs represent a potential source of biomarkers for the early detection and monitoring of tumor responses to treatment and could be utilized for glioblastoma therapy using therapeutic mi-RNA [93]. Arscott et al. showed that irradiation increases the abundance of exosomes released by glioblastoma cell lines and normal astrocytes [97]. These authors showed that exosomes derived from irradiated cells enhanced the migration of recipient cells, and their molecular profiling revealed an abundance of molecules related to signaling pathways important for cell migration, including activation of neurotrophic tyrosine kinase receptor type 1 (TrkA) and FAK signaling [97].

#### Relationship between cell migration and resistance to pro-apoptotic stimuli

Intracellular signaling pathways involved in the acquisition of resistance to pro-apoptotic stimuli by migrating glioma cells, including the PTEN/PI3K/Akt/mTOR and NF- $\kappa$ B pathways, were already an important topic more than 10 years ago [31]. These pathways are not all constitutively activated at the same time in all cells in any one glioma. Many inhibitors of these pathways have been developed, and numerous clinical trials have investigated these agents alone or in conjunction with conventional radiotherapy or chemotherapy. Several recent publications have provided an update on the state of EGFR and PI3K/Akt/mTOR inhibitors in clinical trials for glioblastoma [98,99]. So far, targeted therapy directed to central intracellular signal pathways has not yet achieved satisfactory results in glioblastoma. A future perspective for glioblastoma therapy may be the combined inhibition of multiple targets and more tumor-tailored strategies.

As indicated above, potassium channels may be involved both in the migratory processes and resistance to pro-apoptotic stimuli of glioblastoma cells. A volume change and decrease in intracellular  $\text{K}^+$



**Table 1**  
Potential treatment approaches in clinical study.

Targets	Compounds	Indications	Study	Ref.
Glutamate release by CXC network	Sulfasalazine	Recurrent glioblastoma	Phase I/II	[109]
AMPA receptors	Talampanel	Newly diagnosed glioblastoma	Phase II in association with radio- and temozolomide	[112–114]
NMDA receptors	Memantine	Glioblastoma	Phase I in association with temozolomide	On going
Cl-channel	TM-601	Anaplastic astrocytoma and glioblastoma	Phases I/II and III intracavitary	[46,120]

supported the rationale for the pharmacological targeting of glutamate receptors and transporters in the treatment of glioblastoma ([106] for review). Using the web site <http://www.clinicaltrials.gov/> as a reference, we analyzed the few clinical trials completed with therapies targeting glutamate (see also [107]).

Glutamate release can be inhibited by sulfasalazine, an FDA-approved drug for the treatment of inflammatory bowel disease, through inhibition of the CXC network. This drug may also facilitate death receptor-mediated apoptosis of glioma cells [108]. Sulfasalazine could be considered a therapeutic agent for glioma patients to reduce seizure activity and control invasive growth in patients, although a preliminary study of ten patients with recurrent glioblastoma indicated lack of clinical efficacy [109] (Table 1). Yet, small series of 10 patients are insufficient to draw definitive conclusions about the potential therapeutic benefit of any given drug. Accordingly, Huberfeld and Vecht have discussed the potential for single therapeutic agents, such as the CXC system blocker sulfasalazine or histone deacetylase inhibitor valproic acid, to treat both gliomas and associated epilepsies [110]. However, the same year valproic acid was definitively excluded as a useful therapeutic approach after the analysis of survival of a pooled patient cohort (n = 1869) of four contemporary randomized clinical trials in newly diagnosed glioblastoma patients that received the antiepileptic drug at the start of chemoradiotherapy with temozolomide [111].

Talampanel, an allosteric inhibitor of AMPA receptors, entered phase II clinical trials, and it has been speculated that this drug may provide a survival advantage when used in combination with standard chemoradiotherapy [112,113] (Table 1). However, Grossman and colleagues challenged their own conclusions one year later [114]. Overall survival of 244 patients with newly diagnosed glioblastoma accrued to three consecutive therapeutic trials conducted by the New Approaches to Brain Tumor Therapy (NABTT) CNS Consortium was significantly higher than is reported with radiation and temozolomide alone [30]. The improvement in survival was evident as early as 6 months after study initiation (94% vs 86% survival,  $p < 0.003$ ) and continued over two years (37% vs 27%,  $p < 0.02$ ). In each of these trials a novel agent including talampanel for 72 patients was combined with standard radiation and temozolomide [114]. This report documents significant improvements in the survival of patients with newly diagnosed glioblastoma. However, Grossman et al. emphasized that it remains uncertain if these results are attributable to the novel therapies themselves or reflect evolving patterns of care in this patient population [114]. This argues strongly against the overinterpretation of survival data from uncontrolled phase II studies in newly diagnosed glioblastoma, as has recently been confirmed with the negative outcome of the phase III ACT IV trial [115].

Memantine, a noncompetitive inhibitor of NMDA receptors approved for dementia treatment, is a candidate for modulating the effects of glutamate at the infiltrative rim of gliomas [35,107]. Indeed, a phase I factorial trial of temozolomide, memantine, mefloquine, and metformin for post-radiation therapy glioblastoma is ongoing (<https://clinicaltrials.gov/ct2/show/NCT01430351?term=memantine&cond=glioma&rank=3>) (Table 1).

The goal of this study is to identify the highest tolerable dose of TMZ in combination with a mixture of memantine, mefloquine, and/or metformin that can be administered to glioblastoma patients who have previously undergone radiation and chemotherapy in combination. The

safety of these drug combinations will also be studied (<https://clinicaltrials.gov/ct2/show/NCT01430351?term=memantine&cond=glioma&rank=3>). However, whether such complex trials advance standards of care in glioblastoma remains doubtful, and exploring the addition of high doses of memantine alone to standard chemoradiotherapy might have been preferable. Yohay et al. demonstrated that the local polymer-based delivery of riluzole, a glutamate uptake blocker approved for the treatment of amyotrophic lateral sclerosis, or memantine improved survival in a rat intracranial glioma model when the polymer wafers were placed at the time of tumor engraftment [116].

We speculate that normal neurons might be protected against the high glutamate microenvironment by specific inhibitors of ionotropic receptors but further research is warranted [117,118].

#### Targeting ion channels

One Cl<sup>-</sup> channel inhibitor, TM-601, a synthetic version of the peptide chlorotoxin, a small 36 amino acid neurotoxin isolated from the venom of the giant yellow Israeli scorpion *Leiurus quinquestriatus* [119] that is covalently linked to iodine 131, has completed phase I and II clinical trials for the treatment of anaplastic astrocytomas and glioblastomas through repeated local intracavitary administrations (NCT00591058) [46,120] (Table 1). Intracavitary-administered 131I-TM-601 is well tolerated, remains highly localized to the treatment site, and preliminarily appears safe for repeated injections. As no major toxicity and no death due to 131I-TM-601 have been reported, the FDA allowed the trial to go to Phase III [121]. An FDA approval has also been obtained to investigate the effect of TM601 in newly diagnosed gliomas. TM601 appears to be stable, is not immunogenic and lacks toxicity in humans upon local administration. Parallel to this trial, the same team has worked on an intravenous injection protocol of this product but this time with an imaging application in perspective [122]. However, to our knowledge no study has been published recently. Interestingly, intracranial injection of 131I-CTX detects brain tumors by gamma-ray scintigram scans *in vivo*, but also labels the stomach, indicating that the molecular target of chlorotoxin is also expressed in this organ. So far, TM-601 is the only derivative of chlorotoxin for which human clinical studies have been partially published. Besides the clinical trial of 131I-TM-601, many other chlorotoxin applications have been described that may be potentially useful to treat glioma. A large proportion of them rely on the administration of therapeutic agents, cDNA or siRNA to block oncogene expression, thanks to the use of nanoparticles [123,124].

Molecular targets for chlorotoxin include voltage gated chloride channels, calcium-dependent phospholipid-binding protein annexin-2, and matrix metalloproteinase-2 (MMP-2) [125]. Among these targets, MMP-2 has most anti-neoplastic potential [125].

According to Pardo and Stühmer [48] and our previous study [53], inhibition of potassium channels also has therapeutic potential for glioblastoma patients. Wulf et al. have previously discussed the pharmacological strategies for targeting potassium channels with venom peptides, antibodies or small molecules [126]. The Kv10.1 and Kv11.1 inhibitors have been proposed for the treatment of various types of cancers. Two potent blockers of Kv10.1, the antihistamine, astemizole, and the tricyclic antidepressant, imipramine, have been shown to decrease tumor cell proliferation *in vitro* and, in the case of astemizole,

also *in vivo* [127]. Imipramine binds to the intracellular pocket and consequently inhibits the current of Kv10.1 [128]. Desipramine and imipramine have been demonstrated to inhibit PI3K/Akt/mTOR signaling and induce autophagic cell death *in vitro* in murine C6 and human U87MG glioma cells [129,130].

Recently, Sales et al. showed that *in vitro* suppression of the Kv10.1 channel using astemizole or silencing using a short-hairpin RNA expression vector sensitized U87MG glioma cells to temozolomide [131].

Kv11.1 is affected by drugs that cause acquired long QT syndrome [132]. Even if the intracellular channel pore openings of Kv10.1 and Kv11.1 are similar, although not identical, all of the known Kv10.1 blockers are also effective blockers of Kv11.1 and therefore share cardiac safety problems. For these reasons, the antihistamine astemizole was withdrawn from the market in 2000 [133]. Because the risk benefit profile of an anticancer drug is radically different from that of compounds developed for the treatment of benign conditions, the potential for these compounds should be reconsidered for repositioning. Moreover, there are at least three alternative transcripts of Kv11.1, which are differentially expressed in heart versus tumor cells, suggesting the option of selective inhibition of this type of ion channel in tumors while preserving heart function. A specific peptide toxin that can achieve this result has yet to be reported. However, a monoclonal antibody (mAb56) has been developed that specifically blocks Kv10.1 without affecting Kv11.1 or its close relative Kv10.2 [134]. This antibody showed efficacy *in vitro* against human melanoma MDA-MB-435S and human pancreatic carcinoma AsPC-1 cell lines as well as *in vivo* in nude mice subcutaneously grafted with these tumor models, but the required doses were high, whereas the reduction of tumor growth was modest [134]. The experiments were performed in immunodeficient mice, so that the antibody could, in principle, act exclusively as a channel blocker. Finally, the anticancer compound roscovitine, a cyclin-dependent kinase inhibitor that has terminated phase II clinical trials for non-small cell lung cancer [135], is an efficient blocker of Kv11.1, but does not induce arrhythmia, likely due to its low affinity for closed and inactivated states of the channel.

Staudacher et al. recently identified the Kv11.1 ligand doxazosin as a small molecule compound that promotes apoptosis and exerts anti-proliferative effects in human glioblastoma cells *in vitro* [68]. Doxazosin and future derivatives have therefore been proposed as novel options for more effective glioblastoma treatment [68].

Citalopram (Cipramil®), a selective serotonin reuptake inhibitor used for its antidepressive activity, acts on Kv1.5 currents as an open-channel blocker [53]. Because both imipramine and citalopram have been commonly used to treat depression, which commonly occurs in glioma patients, the European Organization for Research and Treatment of Cancer (EORTC) conducted a large epidemiological study to investigate the actual benefit that these two drugs may provide for glioblastoma patients. D'Alessandro et al. demonstrated that (i) blockade of the calcium-activated potassium channel KCa3.1 with TRAM-34, a high affinity blocker of KCa3.1, exhibits good selectivity for these channels and increases the TMZ antitumor effects, reducing GL-261 glioma cell migration, invasion and colony forming activity, increasing apoptosis and forcing cells to pass the G2/M cell cycle phase, likely through cdc2 de-phosphorylation; (ii) KCa3.1 silencing potentiates the inhibitory effect of TMZ on glioma cell viability; (iii) the combination of TMZ and TRAM-34 attenuates the toxic effects of glioma conditioned medium on neuronal cultures through a microglia-dependent mechanism since the effect is abolished by clodronate-induced microglia killing; (iv) TMZ/TRAM-34 co-treatment increases the number of apoptotic tumor cells and the mean survival time in a syngeneic mouse glioma model (C57BL6 mice implanted with GL261 cells); and (v) TMZ/TRAM-34 co-treatment reduces the cell viability of glioblastoma cells and cancer stem cells freshly isolated from patients [136]. Taken together, these data demonstrate that TMZ/TRAM-34 co-treatment affects both glioma cells and infiltrating microglial cells, resulting in an overall reduction of tumor cell progression. Furthermore, calcium activated potassium

channel blockade by paxilline, a toxin of the fungus *Penicillium paxilli*, inhibited ionizing radiation-induced migration *in vivo* in an orthotopic model of the human glioblastoma model [137]. Additionally, ophiobolin-A, a sesterterpenoid phytotoxin produced by pathogenic fungi of the genus *Bipolaris*, induced marked changes in the dynamic organization of the F-actin cytoskeleton and inhibited proliferation and migration of glioblastoma cells, likely by inhibiting conductance Ca(2+)-activated potassium channel activity [138].

#### Targeting extracellular miRNAs

MicroRNAs are secreted into the extracellular space, at least in part by EV, and are subsequently taken up by normal and cancer cells to sustain and promote tumor growth (Fig. 3). Glioblastoma EVs transport several tumor-specific microRNAs; however, the functions of most of these microRNAs remain unknown. A better understanding of the role of secreted microRNAs may unveil a novel mechanism of glioblastoma progression and resistance to current treatments and may provide novel diagnostic and prognostic biomarkers. Rooj et al. suggested that these biomarkers can be used for the development of targeted therapies aimed at improving glioblastoma patient outcomes [92].

#### Conclusion and perspectives

Glioblastoma is an incurable neoplasm characterized by diffusely infiltrative growth. Given the highly invasive nature of glioblastoma, it remains unlikely that any single therapeutic approach will be able to specifically target and eliminate residual heterogeneous tumor cells. New therapeutic strategies to fight both migrating apoptotic resistant and proliferating glioma cells are necessary to effectively counteract glioblastoma progression. Novel treatment approaches have to integrate the intracellular perspective of the complex intracellular signaling pathways with the microenvironmental orchestral machinery, including connections between heterogeneous tumoral and nontumoral cells, potentially also through channels, microtubules, and extracellular miRNA, generating different messages at different times. New avenues for glioblastoma treatment must not ignore the very specific and peculiar interactions between tumor cells and their host tissue, and the prime targets currently appear to be glutamate- and ion channel-controlled processes.

#### Conflict of interest

The authors declared that there is no conflict of interest.

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