5

The Role of microRNAs in Gliomas and Their Potential Applications for Diagnosis and Treatment

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1. Introduction

MicroRNAs (miRNAs) are a class of single-stranded non-coding RNAs that average 22 nucleotides in length. They regulate gene expression by binding to imperfectly matched sequences in the 3'-untranslated region (3'UTR) of target mRNAs, resulting in either translational repression or destabilization/degradation of their target mRNAs[1-7]. miRNAs play an important role in nearly all cancer types, where they modulate key tumorigenesis processes, such as metastasis, apoptosis, proliferation, and angiogenesis. Each miRNA can affect several different mRNAs and, depending on the target, has the potential to function as an oncogene and/or tumor suppressor.

It has recently been shown that miRNAs play important roles in gliomas [8-19]. A number of miRNAs, such as miR-21, miR-221, miR-222, miR-10b, and the miR-26a cluster [15,19], together with the genes cyclin-dependent kinase 4 (CDK4) and CENTG1, are upregulated in gliomas and appear to act in an oncogenic fashion. Other miRNAs, such as miR-124[8], miR-137[8], miR-34a[12,13], miR-128[16], miR-326[17] and members of the let-7[18] family, are downregulated indicating a tumor suppressive nature.

Several important target mRNAs and pathways have been identified for these onco-miRNAs and tumor suppressor miRNAs. For example, miR-34a is downregulated in glioblastomas as a result of p53 dysfunction, while restoring the expression of miR-34a[12] or miR-7[18] in glioma cells can strongly inhibit oncogenic pathways, such as Ras and Akt.

In addition to the singly transcribed miRNAs, our studies demonstrated that several miRNA clusters were shown to be deregulated in gliomas and might have an important role in the disease process. These clusters comprise the miR17 family, miR183-182, and the SC-specific clusters (miR367-302 and miR371-373), which are upregulated in gliomas. Because the massive cluster of 53 miRNAs on chromosome 14q32.31 is downregulated, it may represent the largest tumor suppressive miRNA cluster[20]. Some of these clusters, such as miR17-92, which is one of the three miRNA clusters related to the miR-17 family, reportedly have a pro-oncogenic roles in other type of cancers[21].

We have recently shown that gliomas share miRNA expression profiles that are similar to neural precursor cells[20]. This result is in agreement with the finding that gliomas contain heterogeneous neoplastic cell populations that phenotypically resemble undifferentiated or immature glial cells.

The study of miRNAs is rapidly developing and could considerably change the current conception of glioma biology. Detecting and quantifying microRNAs in tissue and serum will soon be routinely used for tumor classification and grading as well as diagnostic and prognostic testing for gliomas. For example, high levels of miR-21, miR-182 and miR-196 as well as low expression of miR-181b and miR-106a are associated with poor overall survival in patients with malignant gliomas[22-24], and these miRNAs might function as biomarkers of glioma progression. Furthermore, miR-195, miR-455-3p, miR-10a[25] and the miR-181 family[26] have been linked to treatment resistance, and assessing the relative expression levels of these miRNAs may assist with the design of personalized treatments.

New knowledge regarding the involvement of miRNAs in glioma biology has provided opportunities for the use of miRNAs or their inhibitors as potential candidates for the brain tumor treatments. However, such a therapeutic approach may be considerably challenging in light of several barriers *in vivo,* such as the blood–brain barrier[27], which can prevent miRNAs or their antagonists from entering the brain.

2. MicroRNA regulation of gene expression

miRNAs regulate gene expression inhibition through several proposed mechanisms. One model suggests that there is competition between RISC and eIF4E for mRNA binding, which represses initiation. A second model has proposed that Ago recruits eIF6 and, thus, prevents the association of the 60S ribosomal subunit with the 40S preinitiation complex[28,29]. The third model suggests that miRNAs destabilize target mRNAs by de-adenylation and subsequent decapping[30-34]. It was recently observed that AGO-2, mature miRNAs and translationally repressed mRNAs can accumulate in cytoplasmic processing bodies (P-bodies)[35]. These bodies are enriched with proteins involved in translational repression and in mRNA de-adenylation, decapping and degradation. Thus, they might function not only in storage but also in the decay of repressed mRNAs. However, it remains to be established whether the accumulation of these proteins in P-bodies is the cause rather than the consequence of target mRNA silencing[36,37].

3. Mechanisms responsible for altered miRNA expression in gliomas

Aberrant miRNA expression and function has been frequently observed in gliomas. A number of mechanisms are responsible for altered miRNA expression in cancer:

a. Altered transcription regulation or abnormalities in miRNA processing.

For miR-7, normal pre-miRNA levels were found to be associated with reduced mature miRNA levels in glioblastomas, where it was proven that the reduction in the miR-7 processing was occurred at the pri- to pre-miRNA processing step[38]. Various members of the let-7 family are regulated post-transcriptionally during embryonic brain development, the neural differentiation of embryonic stem cells (SC) and in embryocarcinoma. In these settings, the levels of the pre-miRNAs were consistent during differentiation; however, the

mature forms increased [39]. Few studies shed some light on this mechanism by showing that the let-7 targets, the RNA-binding proteins Lin28 and Lin28B, bind to the loop region of let-7 precursors, which blocks the processing of let-7 at either the Drosha or the Dicer levels[40,41]. This Lin28-mediated degradation of let-7 likely plays a key role not only in development but also in tumorigenesis.

b. *Localization of miRNAs inside or close to cancer-associated genomic regions*[42,43].

This mechanism was suggested also for gliomas based on an array study on human glioma tumors and mouse and human glioma cell lines. The study demonstrated that the majority of the differentially expressed miRNAs were located in regions susceptible to genetic alterations in cancer [20].

c. Epigenetic regulation of miRNA expression.

This is illustrated bychanges in chromatin structures that are induced by covalent modifications of histones and/or DNA methylation[44]. For example, the epigenetic silencing of the miR-124 loci has been observed in brain tumors [8] and precancerous lesions[45]. The epigenetic masking of miR-124 induces activation of the oncogene cyclin-dependent kinase (CDK)-6 and consequent phosphorylation of Rb, resulting in accelerated cell growth.

It has been shown that the Let-7 family is downregulated in gliomas[18]. This miRNA family is considered to be comprised of tumor suppressor miRNAs. The let-7a-3 locus is generally methylated in normal tissues, but it is hypomethylated in some types of cancers. The methylation levels of let-7a-3 correlate inversely with let-7a-3 pri-miRNA expression levels; thus, Let-7a-3 hypomethylation facilitates the epigenetic reactivation of the gene resulting in elevated expression of let-7a-3 and enhanced tumor phenotypes and oncogenic changes[39,46]. Another example is miR-128, which is not regulated by epigenetics, but has been shown to play a role in the epigenesis of glioma SCs. Its downregulation in glioma tissue causes the elevated expression of Bmi-1, one of the polycomb group of genes that function as epigenetic silencers, which enhances cancer stem cell self-renewal through chromatin remodeling[16].

4. The involvement of miRNAs in pathways that may promote gliomagenesis and tumor progression

miRNAs have been shown to act as both oncogenes or tumor suppressor genes and, thus, affect pathways that bestow nearly all hallmarks of cancer[47,48]. These miRNAs can, in turn, promote gliomagenesis and tumor progression.

a. Sustaining proliferative signaling

For a tumor to become independent from external growth factor signals, it requires the activation of different cell proliferation and survival pathways, such as those mediated by epidermal growth factor receptor (EGFR) and Akt, which play a central role in glioblastomas. It has been demonstrated that miR-7 directly inhibits the expression of EGFR[38,49] and its downstream effector, Raf1, and suppresses the AKT pathway by targeting upstream regulators. Indeed, transfection with "mimic" miR-7 oligonucleotides decreased viability and invasiveness and induces cell cycle arrest and apoptosis of glioblastoma cell lines[38,49].

The altered regulation/activation of RAS proteins, which process signals downstream of growth receptors, plays a key role in the deregulation of multiple proliferation pathways in most types of tumors, including gliomas. Decreased levels of let-7 in gliomas correlate inversely with overexpression of RAS proteins, while restoring let-7 expression reduces the expression of RAS in glioma cell lines resulting in tumor growth inhibition *in vitro* and *in vivo*[18]. Moreover, the activation/overexpression of RAS leads to the upregulation of miR-21 *in-vitro* and *in-vivo*. Mir-21 exerts its oncogenic effect by downregulating the phosphatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4)[50]. In a number of human glioblastoma cell lines, such as T98G, A172, U87, and U251, the expression of PDCD4 protein correlates inversely with expression of miR-21. The downregulation of miR-21 in those cell lines leads to decreased proliferation, increased apoptosis, and decreased colony formation on soft agar[51].

DNA amplification of an onco-amplicon was observed in a subset of high-grade gliomas. This onco-amplicon consists of miR26a-2 and the oncoproteins CDK4 and CENTG1, which regulate the RB1 and PI3 kinase/AKT pathways, respectively. miR-26a alone can functionally target PTEN, RB1, and MAP3K2/MEKK2 protein expression, thereby increasing AKT activation, promoting proliferation, and decreasing c-JUN N-terminal kinase-dependent apoptosis *in vitro* and *in vivo*. The overexpression of miR-26a in cells overexpressing CDK4 or CENTG1 further promotes tumor growth *in vivo*. Glioblastoma patients harboring this amplification display markedly decreased survival[15,19].

b. Evading growth suppressors

miRNAs may also affect cell proliferation by controlling cell cycle regulators. The E2Fs transcription factor family plays a pivotal role in cell cycle progression. In response to mitogenic signaling, pRB is sequentially phosphorylated by the CDK/cyclin complexes leading to activation of E2F-responsive genes to promote cell cycle progression[52]. This pathway can be inhibited by several miRNAs. For example, miR-137, and miR-124a inhibit CDK6 expression in different cancer cell lines, including gliomas[8], where the transfection of miR-124 or miR-137 has been shown to prevent cell cycle progression in glioblastoma cell lines, which is associated with the decreased expression of CDK6 and pRB proteins[8]. miR-34a, which is transcriptionally activated by p53, can target both CDK6 and cyclin D1 preventing the downstream pro-survival signaling of the cyclin/CDK pathway. Restoring the expression of the underexpressed miR-34a in glioma cell lines downregulates CDK6 protein expressions and inhibits cell proliferation.[12]

The negative regulators of the cyclin/CDK pathway, such as members of the Cip/Kip family (p21, p27 and p57) and the INK4a/ARF family (p14 and p16), are regulated by several miRNAs. Upregulation of these miRNAs may inhibit these negative regulators and result in the proliferation and survival of the cells.

The miRNA clusters miR-106b-25 and miR-17-92 have anti-proliferative and pro-apoptotic activities in different tumor types through the inhibition of p21 in addition to other genes. Although they are also upregulated in gliomas[20], their role in the inhibition of p21 or other negative regulators of cyclin/CDK remain to be elucidated. High levels of miR-221/222, which targets both p27 and p57, appear in glioblastomas. Functional studies showed that miR-221/222 prevents quiescence when elevated during growth factor deprivation and induces precocious S-phase entry, thereby triggering cell death[53]. In addition, the

overexpression of these miRNAs increases glioma cell proliferation *in-vitro* and induces glioma growth in a subcutaneous mouse model[54]. The inhibition of miR-10b, which is strongly upregulated in gliomas, reduces glioma cell growth through cell-cycle arrest and apoptosis. These cellular responses are mediated by augmented expression of its direct targets, including p21 and CDKN2A/p16[55].

c. Resisting cell death

In addition to the role of miR-221/222 in cell growth and cell cycle progression, it has been demonstrated that miR-221/222 directly regulates apoptosis by targeting p53-upregulated-modulator-of-apoptosis (PUMA) in glioblastoma. PUMA binds to Bcl-2 and Bcl-XL through a BH3 domain and the exogenous expression of PUMA results in an extremely rapid and profound apoptosis[56]. Thus, the forced expression of miR-221/222 downregulates PUMA and induces cell survival, whereas the knockdown of miR-221/222 induces PUMA expression and cell apoptosis as well as decreases tumor growth in a xenograft model[57].

d. Inducing angiogenesis

Recent studies have revealed important roles for miRNAs such as the endothelial cell (EC)-restricted miRNA miR-126 as well as miR-378, miR-296, miR-92a and the miR-17-92 cluster in regulating angiogenesis. Thus, these have been termed angiomirs[58].

The level of the angiomir miR-296, which inhibits the degradation of the VEGF receptor, is increased in EC cells co-cultured with glioma cells or in response to angiogenic growth factors (including VEGF). When miR-296 is inhibited *in vivo*, the vascularization of tumor xenografts decreases[59]. miR-93, one of the miRNAs within the miR-106b-25 cluster, a member of the miR-17 family, enhances cell survival, promotes sphere formation and augments tumor growth. *In vivo* studies revealed that miR-93-expressing cells induced blood vessel formation, likely through targeting integrin-β8, allowing blood vessels to extend to tumor tissues in high densities. These findings show that miR-93 promotes tumor growth and angiogenesis through the suppression of integrin-β8 expression.

e. Activating invasion and metastasis

As mentioned above, miR-21 is overexpressed in gliomas. The metalloproteinase (MMPS) inhibitors RECK and TIMP3 are two targets of miR-21. RECK is a membrane-anchorage regulator, while TIMP3 is an extracellular matrix (ECM)-bound protease inhibitor. Treatment with antisense oligonucleotides to miR-21 in glioma cell lines and in a nude mouse model of human glioma resulted in elevated levels of RECK and TIMP3 and, therefore, reduced MMP activities. Thus, downregulation of miR-21 in glioma cells leads to a decrease in their migratory and invasion abilities[11].

miR-10b is highly expressed in many tumors, including glioblastomas. It was found that miR-10b induces glioma cell invasion by modulating the expression of the tumor invasion factors MMP-14 and uPAR through directly targeting HOXD10. Accordingly, glioma cells lost their invasive ability when they were treated with specific antisense oligonucleotides to miR-10b.

Not surprisingly, additional miRNAs are currently being identified and associated with ECM reorganization in relationship to cancer. Like miR-21 and 10b, miR-146b has also been demonstrated to play a role in glioma cell invasion[60]. However, miR-146b does not function

through the suppression of ECM inhibitors; rather, its loss in gliomas allows the upregulation of MMP16, which might cause proteolysis of ECM components, such as type III collagen. Thus, low levels of miR-146b contribute to the migration and invasion of glioma[60].

f. Reprogramming energy metabolism

Pyruvate kinase type M2 (PKM2) is one of four isoenzymes of pyruvate kinase, which catalyzes the last step within glycolysis. This enzyme is normally thought to be embryonically restricted; however, it has been shown to be expressed in cancerous cells[61]. Recently, it was shown that the levels of PKM2 negatively correlate with the levels of miR-326, suggesting a regulatory relationship between PKM2 and miR-326. Furthermore, miR-326 decreased the glioma metabolic activity by decreasing ATP levels, suggesting that miR-326 could regulate glioma metabolism through the downregulation of PKM2[62].

5. The glioma microRNA expression signature

Differential miRNA expression in gliomas was first reported by Ciafre`[63] and Chan[64]. Their study revealed that miR-21[64] and miR-221 are upregulated, whereas miR-128, miR-181a, miR-181b and miR-181c are downregulated, in glioblastoma[63]. Other array-based approaches identified that the expression of miR-124, miR-128a, and miR-137 are decreased in anaplastic astrocytomas and glioblastomas[8]. In addition, miR-124 is decreased in oligodendroglial tumors[65] relative to non-neoplastic control brain tissues.

The expression levels of miR-124 and miR-137 were increased during the differentiation of mouse neural progenitor cells (NPCs) following growth factor withdrawal. Thus, the authors have suggested that these specific miRNAs display "stemness" of glioma cells and that alteration of the levels of these miRNAs may induce differentiation[8].

While these studies compared microRNA expression in gliomas relative to control tissues, other studies explored the role of miRNAs in glioma progression. One study investigated miRNA expression profiles in primary WHO grade II gliomas that spontaneously progressed to WHO grade IV secondary glioblastomas. They identified 12 miRNAs (miR-9, miR-15a, miR-16, miR-17, miR-19a, miR-20a, miR-21, miR-25, miR-28, miR-130b, miR-140 and miR-210) that were upregulated and two miRNAs (miR-184 and miR-328) that were downregulated upon glioma progression[66]. Other studies have compared the differential miRNA expression between astrocytomas and glioblastomas and revealed a 23-miRNA expression signature that can discriminate glioblastomas from anaplastic astrocytomas with an overall diagnostic accuracy of 89.7%67.

Our study compared the miRNA expression signatures of glial tumors, embryonic SCs, NPCs and normal adult brains from both human and mouse tissues. We demonstrated that all gliomas displayed NPC-like miRNA signatures. About half of the miRNAs expressed in the NPCs-glioma shared profile were clustered in seven genomic regions. These clusters comprised the miR17 family (3 clusters), miR183-182, and the SC-specific clusters miR367-302 and miR371-373, which are upregulate. They also contained the bipartite cluster of 7+46 miRNAs on chromosome 14q32.31, which is downregulated in the shared expression profile. These seven regions are particularly prone to genetic and/or epigenetic aberrations

in different types of cancers (e.g., LOH on chromosome 14q32.31, or the amplification of chromosome 13q31.3). Together, these findings suggest that NPCs may be the originating cells in gliomas and that aberrations in critical regions might be necessary to maintain the stem cell nature of gliomas[20].

A recent paper analyzed microRNA expression data from The Cancer Genome Atlas (TCGA) and identified five clinically and genetically distinct glioblastoma subclasses related to a different neural precursor cell types. Thus, like us they also suggested that glioblastomas can arise from neural precursor cells but emphasized that the cell of origin could arise from multiple stages of differentiation[68].

6. microRNAs as biomarkers for the diagnosis and prognosis of gliomas

As reviewed above, a large number of recent studies have discovered that miRNAs play important regulatory roles in a variety of cellular functions in gliomas. Other papers have demonstrated that miRNAs can be used for tumor classification and grading[66,67] in addition to the diagnosis[23], prognosis[22,23] and prediction of therapeutic efficacy[69, 69] of tumors. The discovery of miRNAs in the serum of cancer patients[70] opened up the exciting possibility of using miRNAs as non-invasive biomarkers. Thus, detecting and quantifying microRNAs may soon be used in routine clinical practice for diagnostic and prognostic glioma testing.

The expression levels of miR-182[22] and miR-196[23] are significant in correlation with World Health Organization glioma grading ($P < 0.001$). Multivariate analysis showed that the expression of both these miRNAs is a predictor of overall survival in glioblastoma patients [22,23]. Analysis of miRNA expression data in glioblastoma patients (n = 222) derived from the TCGA dataset identified an expression signature of ten miRNAs that can predict glioblastoma (GBM) patient survival[71]. In astrocytomas, the downregulation of miR-137 has been shown to be associated with advanced clinical stages of the disease; and the low expression levels of miR-181b and miR-106a, or high expression of miR-21, are significantly associated with poor patient survival[24]. Several papers aimed to identify the miRNAs specifically involved in the acquisition of temozolomide (TMZ) resistance in glioblastoma. In one article, the authors established resistant variant U251R cells from a TMZ-sensitive glioblastoma cell line (U251MG) and performed miRNA microarray on both the resistant and sensitive cell lines. The results showed that miR-195, miR-455-3p and miR-10a* were the three most upregulated miRNAs in the resistant cells. When miR-195 was reduced, the resistant cells displayed a moderate cell killing effect, and the combination with TMZ strongly enhanced this effect[25]. Another article examined the correlation between the expression levels of selected microRNAs in 22 primary glioblastomas with response to concomitant therapy (e.g., chemoradiotherapy with TMZ). They found that miR-181b and miR-181c were significantly downregulated in patients who responded to concomitant therapy compared to patients with progredient disease[26].

Over many decades, it has been shown that cell-free DNA and RNA is present in the circulation and may represent potential biomarkers. We have demonstrated that tumor-specific DNA can be detected in the serum of glioma patients and is a potentially promising tool for brain tumor diagnosis[72]. Skog et al. was the first to demonstrate that

brain microvascular endothelial cells take up exosomes, which contain mRNA, miRNA, and angiogenic proteins released by glioblastoma cells. Moreover, they showed that miR-21 is more elevated in serum microvesicles from glioblastoma patients than in healthy controls[73].

Based on their potential as prognostic and diagnostic biomarkers, circulating miRNAs are promising non-invasive tumor markers. Tumor-specific circulating miRNAs may improve cancer diagnosis and prognosis because, as described above, several promising miRNAs have already been recognized as potential biomarkers for gliomas.

7. The therapeutic potential of microRNAs in gliomas

With the increased understanding of the miRNA target genes, the cellular behaviors influenced by them and the ability of one microRNA, such as let-7 or miR-21, to target more than one gene or pathway provide exciting opportunities to use miRNAs or their inhibitors as potential candidates for the treatment of brain tumors.

There have been few pre-clinical and phase I/II studies that showed some success in using synthetic miRNA mimics or anti-miRNA oligonucleotides as therapeutic agents. Two of these studies demonstrated that delivery of the locked-nucleic-acid (LNA)-anti-miR in African green monkeys silences miR-122, decreases the total plasma cholesterol[74] and suppresses HCV viremia[75] with no evidence of hepatotoxicity. Another study showed that the silencing of miR-155 in a mouse inflammation model by LNA-anti-miR administration results in derepression of the C/EBP Beta isoforms and downregulation of granulocyte-colony stimulating factor expression in mouse splenocytes[76]. In gliomas, the silencing of miR-21 by LNA-anti-miR followed by TRAIL treatment increased caspase activity *in vitro* and reduced tumor growth *in vivo*[10]. These results support the potential of LNA-anti-miR as therapeutics for inhibition of disease-associated miRNAs.

An alternative to chemically modified antisense oligonucleotides is the "miR sponge". These competitive miR inhibitors contain multiple binding sites to an miR of interest, preventing it from binding to its natural target. In this way, a single sponge can block all miR family members containing the same seed sequences. The sponge strategy has been used to inhibit miR-31 *in vivo* in a noninvasive breast cancer cell line. miR sponges that carried miR-31 recognition motifs were introduced into a retroviral vector and reduced miR-31 function significantly[77].

Some preclinical studies have shown promising results using self-complementary adeno-associated viral (scAAV) vectors. For example, a scAAV vector containing miR-26a was administered with a single tail-vein injection into mice with established liver tumors. High miR-26a levels were found in their livers and no toxic effects were observed. While six out of eight mice treated with the control virus developed tumors, eight out of ten miR-26a-treated mice developed only small tumors or had a complete absence of tumors[78].

Although most of these therapeutic approaches appear promising for systemic tumors, they would be considerably challenging in brain tumors due to several obstacles and barriers *in vivo,* such as the blood–brain barrier[27], which might prevent the miRNAs or their antagonists from entering the brain. Thus, the development of more efficient and specific

delivery systems is necessary before miRNAs can be used as therapeutic agents for the treatment of malignant gliomas.

At a glance:

- A number of miRNAs are upregulated in gliomas and appear to act as oncogenes while other miRNAs, are downregulated indicating a tumor suppressive nature. These onco-miRNAs and tumor suppressor miRNAs modulate key processes that promote gliomagenesis and tumor progression, such as sustaining proliferative signaling, evading growth suppressors, resisting cell death, inducing angiogenesis, activating invasion and metastasis and reprogramming energy metabolism
- Not only individual, but also miRNA clusters are deregulated in gliomas. Some, such the huge cluster of 53 miRNAs on chromosome 14q32.31 are downregulated in gliomas, and may act as a tumor suppressive miRNA clusters and others such as miR17-92 are upregulated and reportedly have a pro-oncogenic role in cancer.
- Gliomas display miRNA expression profile that is similar to neural precursor cells. This result is compatible with the phenotypically resemble of part of the neoplastic cell populations within glioblastomas to undifferentiated or immature glial cells.
- Detecting and quantifying microRNAs in tissue and serum will soon be routinely used for tumor classification and grading as well as diagnostic and prognostic testing for gliomas and may assist with the design of personalized treatments.
- Manipulating the expression of miRNAs that are involved in glioma biology might provide opportunities for brain tumor treatments.

8. References

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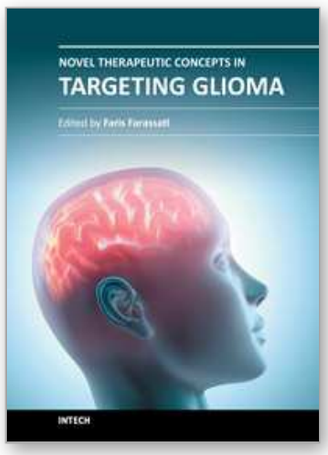

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Novel Therapeutic Concepts for Targeting Glioma offers a comprehensive collection of current information and the upcoming possibilities for designing new therapies for Glioma by an array of experts ranging from Cell Biologists to Oncologists and Neurosurgeons. A variety of topics cover therapeutic strategies based on Cell Signaling, Gene Therapy, Drug Therapy and Surgical methods providing the reader with a unique opportunity to expand and advance his knowledge of the field.

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