IDENTIFICATION OF MICRORNAS INFLUENTIAL IN GLIOBLASTOMA CANCER STEM CELLS

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LIST OF ABBREVIATIONS

GBM	Glioblastoma Multiforme
OFS	l'Office Fédérale de la Statistique
TP53	53kd-protein acting as a tumor suppressor gene; "genome's guardian"
RB	Retinoblastoma Protein
PDGFRA	Platelet-derived Growth Factor Receptor, Alpha Polypeptide
CDKN2A	Cyclin-dependent Kinase 2A
P16	Cyclin-dependent Kinase Inhibitor 2A
MDM	Mouse Double Minute Homolog
MMP	MATRIX metalloprotease
FGF	Fibroblast Growth Factor
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet-derived Growth Factor
LOH	Loss of Heterozygosity
PTEN	Phosphatase and tensin homologu
EGFR	Epidermal Growth Factor Receptor
Hif1α	Hypoxia-inducible Factor 1-alpha
P18	Cyclin-dependent Kinase 4 Inhibitor C
CDK4	Cyclin-dependent Kinase 4
Arf	Alternate Reading Frame Protein
IDH1	Isocitrate dehyodrgenase
PI-3 Kinase	Phosphatidyl-inositol-3 Kinase
MGMT	O^6-methylguanine DNA methyltransferase
BT	Brain Tumor
MiR	microRNA
mRNA	messengerRNA
GFAP	Glial Fibrillary Acidic Protein
NES	Nestin
NF1	Neurofibromatosis Type 1
Akt (PKB)	Protein Kinase B
TNF	Tumor Necrosis Factor
NF-κB	Nuclear factor Kappa-light-chain-enhancer of activated B cells
MERTK	Proto-conogene tyrosine—protein kinase MER
CHI3L1	Chitinase-3-like protein 1
MET (HGFR)	Hepatocyte Growth Factor Recepto
PIK3CA:	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
SOX	Superfamily of genes related to SRY (Sex determing Region on Y)
DCX	Doublecortin protein encoded by the gene DCX
DLL3	Delta-Like 3
ASCL1	Achaete-scute Homolog 1
TCF4	Transcription Factor 4
NKX2-2	Transcription factor involved in morphogenesis of the nervous system
OLIG2	Oligodendrocytes transcription factor
P21 (CDKN1A)	Cyclin-dependent Kinase Inhibitor 1A
NEFL	Neurofilament Light Polypeptide
GABRA1	Gamma-Aminobutyric Acid Receptor Alpha1
SYT1	Synaptotagmin 1
SLC12A5	Solute carrier family 12 (K1/Cl- transporter), member 5
WHO	World Health Organization
Ki-67	Antigen for proliferation. Kiel (Germany), 96-well plate 67

LIST OF ABBREVIATIONS (2)

MIB-1	Antigen for proliferation
Gy	Gray
CCNU	Lomustine, chemotherapy
GSC	Glioma Stem Cells
CD133	cluster of differentiation 133
LCAM1	L1 Adhesion Molecule
SSEA-1	Stage-specific embryonic antigen 1
CXCL12	chemokine (C-X-C motif) ligand 1
CXCR4	C-X-C chemokine receptor type 4
NSAID	Non-Steroidal Anti-Inflamatory Drugs
UTR	Untranslated Region
Pri-miR	Primary microRNA
Pre-miR	Precursor microRNA
DGCR8	DiGeroge Syndrome Chromosomal Region 8
dsRNA	Double-Stranded RNA
RISC	RNA-induced silencing complex
AGO	Agonaut
XPO5	Exportin5
ATM	Ataxia Telangiectasia Mutated
C. Elegans	Caenorhabditis Elegans
SMAD	“Mothers Aganais Decapentaplegic” + SMA in C. Elegans
TGFβ	Transforming Growth Factor Beta
BMP	Bone Morphogenetic Protein
TRBP	TAR (transactivation response) RNA-Binding Protein
r-SBE	TGFβ/BMP-regulated miRNA consensus sequence
Oct4	Octamen Binding Transcription factor 4
Nanog	From Scottish “Tìr na nÒg Legend” meaning “Land of Youth”
TMEM49	Transmembrane Protein 49
RECK	Matrix metalloprotease regulator
TIMP3	Tissue Metallopeptidase Inhibitor 3
IGFBP3	Insulin-Like Growth Factor-Binding Protein 3
Bax	Bcl-2-like-Protein 4
Bcl-2	B-cell Lymphoma 2
NOD-SCID commonγKO	Non-Obese Diabetic, Severe Combined Immunodeficiency, Knock-Out
LNA	Lockec Nucleid Acid
BRDU	5-bromo-2’-deoxyuridine
SVZ	Subventricular Zone
Scr-Ctrl	Scrambled Control
FASLG	Fas (transmembrane protein) Ligand
GFP	Green Fluorescent Protein
IRB	Institutional Review Board
PenStrep	Penicillin Streptomycin
NEAA	Non-Essential Amino Acid
FCS	Fetal Calf Serum
M-MLV	Moloney Murine Leukemia Virus
PPIA	Peptidylprolyl Isomerase A (Cyclophilin A)
SNORD49a	Small Nulceolar RNA, C/D Box 49A
ACK	Ammonium-Chloride-Potassium
HEK	Human Embryonic Kidney
CMV	Cytomegalovirus

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1. INTRODUCTION

1.1 BACKGROUND ON GLIOBLASTOMA

1.1.1 EPIDEMIOLOGY

Cerebral tumors account for 1-2% of all cancers in Switzerland(1)(2). 58% of primary brain tumors are gliomas (1). Of the 500-700 (2) persons suffering from gliomas each year in Switzerland, 250-300(3) have glioblastoma multiforme (GBM), which makes it the most common type of malignant primary brain tumor in adults, whereas cerebral metastases represent the majority of all brain tumors (4). Caucasian males living in industrial areas are believed to be most often affected by GBM (5)(6). Despite its controversial role in GBM pathogenesis, l'Office Fédéral de la Statistique (OFS) recommends limiting radiation from cell phone use (1).

1.1.2 CLINICAL MANIFESTATIONS

Clinical manifestations of GBM are variable — depending on the tumor's localization and rate of progression — and span a range of diverse signs and symptoms. However, the first manifestation of GBM is often a seizure, accompanied by a long-lasting atypical headache and a focal neurological deficit (7). General symptoms, such as nausea, vomiting, and loss of appetite, caused by increased intracranial pressure due to the tumor's mass surrounded by oedema are common (1). GBM rarely metastatizes, even though spreading through the subarachnoidal space to the spinal cord can occur (7). Its principal location is in the subcortical hemispheres, involving white matter (7) and, typically, fronto-temporal location is a predilection site (8). Clinical history is usually short, less than three months in 50% of patients affected by de novo GBM. However, secondary GBM, which accounts for 40% of cases (9) and arises from grade II or III gliomas, may have subtle manifestations for one-to-ten years before being diagnosed. It is often impossible to distinguish between a benign lesion and a malignant tumor based on the clinical history alone (10).

1.1.3 PATHOGENESIS

HISTOLOGY

Glia, the neuronal support tissue can occasionally give rise to gliomas, which are divided into astrocytomas, oligodendrogliomas or ependymomas, depending on the cell of origin. Gliomas are categorized into four different grades, whose characteristics are summarized in **Figure 1**. Notably, GBM defines grade IV astrocytomas and is the most aggressive type of glioma.

	Cell of origin	Grade		Name	Macroscopy (7)	Microscopy (7)	Survival rate (2)
Astrocytoma	Astrocytes	*Non-infiltrating*	I	Pilocytic Astrocytoma	Cystic predominantly	Bipolar cells with GFAP+ processes, Rosenthal fibers, eosinophilic granular bodies, necrosis and mitoses are uncommon	Forseeable recovery if total resection
		Infiltrating	II	Diffuse Astrocytoma	Poorly defined, gray, infiltrative, cystic degeneration may be present	Increased glial cellularity, variable nuclear pleo-morphism, GFAP+ astrocytes processes-> fibrillary aspect	7-10y
			II	Anaplastic Astrocytoma	Similar	More densely cellular, more nuclear pleomorphism	3,5y
			IV	Glioblastoma	Variation is specific: firm/white, soft/yellow/necroti c, cyst and even hemorrage	Similar to anaplastic +endothelial proliferation (double layer, glomeruloid body), pseudopalisades surrounding necrosis	Methylated: 23m Non-methylated: 13m
Oligodendroglioma	Oligodendcocyts	Lower grade Often II/IV			Well-circumbscribed, gelatinous, gray +cysts, hemorrhage, calcification	Regular cells with spheroical nuclei with finely granular chromatin Perineuronal satellitosis	5-10y
		Higher grade Often III/IV		Anaplastic		Increased cellular density, nuclear anaplasia, increased mitotic activity, necrosis, confusable with GBM	
Ependymoma	Ependymal cells	Several names depending on the location			Solid or papillary masses	Regular, round/oval nuclei, abundant granular chromatin, perivascular pseudorosettes, GFAP+	20-30% à 5ans (11)

Figure 1: Classification and description of gliomas (12)

SIGNALING PATHWAYS

The majority of GBMs are believed to develop sporadically, though specific genetic alterations are progressively coming to light. Transformation of a permissive cell by one of several possible oncogenic events provides growth and survival advantage that lead to the emergence of a clone, which retains the features of the initial transformed cell. The clone then progressively evolves into a heterogeneous tumor mass through a combination of genetic and epigenetic modifications. The cell of origin of GBM remains unidentified although adult neural progenitor and mature glial cells are proposed to be plausible candidates (13).

In GBM, *TP53, RB* and receptor tyrosine kinase pathways are commonly altered (10). Lower grade astrocytomas are associated with mutation of *TP53*, which hinders DNA repair, and overexpression of *PDGFRA.* Transition to a higher grade ordinarily implies deletion or suppression of two tumor suppressor genes *p16/CDKN2a* and *RB* (7) along with dysregulation of *MDM2, MDM4* and *MMP9* expression and the upregulation of several growth factors such as FGF, VEGF and PDGF1. Interestingly, mutation and dysregulation of *Ras* and *PDGFβ* lead to oligodendroglioma development (13).

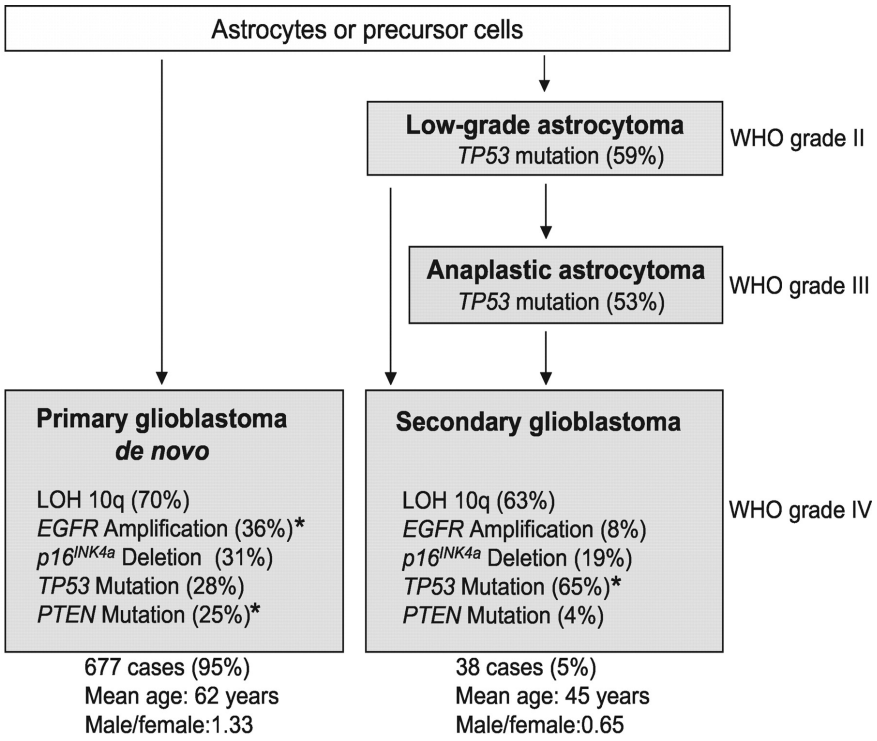


Figure 2: Time and frequency of genetic alterations during astrocytoma progression. Reproduced from (9)

Major events in GBM tumorigenesis are (13):

- **Differentiation**: Tumor progenitor cells show phenotypic properties of either astrocytoma or oligodendroglioma depending on the oncogenic hits on one hand and the microenvironment on the other, leading to expression of neural and glial markers and involving different pathways. Commonly, dysregulation of *p53* leads to an astrocytoma, whereas oligodendrogliomas display chromosome 1p19q translocation.
- **Necrosis/hypoxia**: Highly malignant tumors have high metabolic demand. Nevertheless, intratumoral vasculature is leaky and inclined to develop thrombosis, leading to hypoxia and central necrosis. Hypoxia activates *Hif1α,* which increases migration and gelatinase activity, as well as secretion of growth factors. This state selects apoptotic resistant tumor cells bearing inactive *p53,* which appear to be more aggressive, heterogenous and resistant to cytotoxic therapies.
- **Angiogenesis**: Microvascular proliferation occurs via VEGF, leading to proliferation of endothelial and smooth muscle cells and formation of glomeruloid vessels. These structures are irregularly distributed, in a semi-circular pattern around necrotic foci and sometimes at invasive edges. VEGF and PDGR secretion can participate in the breakdown of the blood/brain-barrier, leading to increased permeability and therefore oedema.
- **Invasion** occurs by exhibiting, on a histopathological level, secondary structures of Scherer, which affect white matter. Sometimes butterfly aspects arise involving both hemispheres, but perineuronal, subglial and perivascular spaces are engaged. Key functions deregulated are: cell adhesion, motility in the extracellular matrix and signal

transduction pathways. Brain matrix consists of mostly hyaluronic acid, with basal lamina in perivascular regions. Effector molecules implicated include metalloproteases, such as MMP2 and MMP9, integrins and growth factors including FGF, EGF and VEGF. Amplification of *EGFR* pathways is found in cells on the infiltrative edges and is believed to play a role in recurrence.

- **Tumor progression**: In 50% of GBM, the cell cycle is deregulated by involvement of *p18, CDK4, CDK6, cyclinD1* and *Rb*. The other 50%, *CDKN2A,* which encodes p16 and Arf proteins, is homologously deleted, mutated or hypermethylated. Primary GBMs that show aggressive outcome, harbor amplification of *MDM2* and aberrant forms of EGFR*,* such as *EGFRvIII* (mutated form lacking exons 2-7) or *v-ErbB* (present in 30% of GBM) and display loss of *PTEN* (14). On the other hand, secondary GBM usually have *p53* mutated, loss of chromosome 19q (14) and increased signaling via *PDGFRA. IDH1* mutations also occur in a majority of secondary GBM (14). Nonetheless, both pathogenic pathways lead to activation of *RAS* and PI-3 kinase, as well as increased receptor tyrosine kinase activity, that result in tumor growth (7). Loss of 1p and 19q chromosomes, which are typically signatures for an oligodendroglial phenotype, have been found in a considerable fraction of GBMs that have a better survival rate (22.2m vs. 13m) (12).

MOLECULAR SUBTYPES

When focusing on pathogenesis on a transcriptome level, GBM is divided into 4 different classes: classical, proneural, neural and mesenchymal. Each category's specificities are compiled in **Figure 3**. Customarily, there is no switching between subclasses when recurrence of GBM occurs. Additionally, no association has been made regarding the DNA repair gene *MGMT's* methylation, which is an important prognostic factor, and subtypes (15). Brain Tumors (BT) 4, 8 and 11, all primary cell cultures that we have used, have been classified as proneural GBM cells after RNA-seq analysis.

Classification based on microRNA (miR) expression profiles appears to be even more precise than that based on messengerRNA (mRNA) expression. On this basis, five GBM clusters have been identified and are correlated to different clinical outcomes. Thus, expression of miR196a/b, among 10 other miRs, is associated with shorter overall survival (16). MiR expression profiles have not only been used to differentiate GBM from anaplastic gliomas, but have enabled us to identify these 5 subclasses of GBM (17). However physical interactions between miRs and mRNAs have to be better understood in order to gain in classification precision.

Subtype	Genetic alterations	Possible progenitor cell	Survival (months)	Clinical specificity	Mean age	MiR identification (18)
Classical	Chr7 ampl, loss of chr10, lack of *TP53* mut, *Ink4a/ARF* del, ***EGFR*** ampl + alt If *EGFR* ampl, Rb pathway altered via *CDKN2A* del Notch, Sonic hedgehog pathways and neural precursor and stem cell marker *NES* highly expressed	Astrocytic signature	12.2	More advantage regarding mortality if treated aggressively *	55.7	Risky : miR26a, miR767, miR153, miR31, miR22 Protective : miR654, miR422b
Mesenchymal	***NF1*** del + mut + co-mut with *PTEN* interfering on AKT pathway TNF and NK-κB pathway highly expressed EMT due to higher activity of mesenchymal and astrocytic markers such as *CD44* and *MERTK* Mesenchymal *CHI3L1* and *MET,* Swann cell S100A, as well as microglial markers expressed	Astroglial + microglial signature	11.8	Higher necrosis rate and inflammatory infiltrates More advantage regarding mortality if treated aggressively * Link via *NF1* with neurofibromas ?	57.7	Risky : miR373, miR296, miR191 ,miR602 Protective : miR223
Proneural	***IDH1*** mutation or ***PDGFRA*** ampl + mut in Ig-domain, *TP53* mut +LOH If no *PDGFRA* abnormality, *PIK3CA*/*PIK2R1* mutated High expression of proneural development genes: *SOX, DCX, DLL3, ASCL1 and TCF4* High expression of oligodendrocytic genes: *PDGFRA, NKX2-2, OLIG2.* *OLIG2* downregulates p21 and, thereby, *CDKN1A*	Oligodendrocytic signature	11.3	No use of aggressive* treatment More inclined to recurrence Possibly secondary** GBM Resemblance with oligodendro-gliomas	51.8	Risky : miR582 Protective : miR130, miR195 NonG-CIMP Risky : miR335, miR34a, miR581, miR21 Protective : miR361, miR145, miR143, miR378, miR182, miR183
Neural	Neuron markers *NEFL, GABRA1, SYT1, SLC12A5* characteristic	Oligodendrocytic and astrocytic Neurons	13.1	Similar expression pattern with normal brain tissue	63.8	Risky : miR222 Protective : miR422a, miR662, miR566, miR24, miR370, miR492, miR629

Figure 3: Molecular description of GBM subtypes. Genes in bold are those defining each category (15). *Aggressive stands for either a concurrent radiochemotherapy or at least three subsequent chemotherapy cycles ** stemming from grade II or III glioma. Ampl = amplification, chr = chromosome, alt = alteration, del = deletion, mut = mutation, EMT= epithelial-to-mesenchymal transition, LOH = loss of heterozygosity

1.1.4 Diagnosis

Definite diagnosis is confirmed after histopathological confirmation. Strong clinical suspicion, supported by radiological findings will encourage a stereotaxic biopsy or even resection. Usually fresh frozen sections are performed intra-operatively to guide the neurosurgeon in the sampling and give the most accurate intra-operative diagnostic interpretation. Cytopathology, immunohistochemistry and genetic assessment are the most employed tools to determine precisely the lesion's nature and, in the case of a glioma, grade it according to the World Health Organization (WHO) classification (12). Usually hematoxylin and eosin stains are used in histopathology, glial fibrillary acidic protein (GFAP) and cytokeratin are relevant markers and Ki-67/MIB-1 help determine the cell proliferation rate. Expression of GFAP is usually associated with astrocytic tumors, whereas loss of 1p and 19q chromosomes underlies oligodendroglial phenotypes (19). Unfortunately, despite establishment of precise criteria, inter- (20) and intra-tumoral (21)(22) heterogeneity of GBM renders classification challenging. Thus, an important debate is still ongoing about how to name a glioblastoma with oligodendroglial component; "oligoastroyctoma WHO grade IV" or "oligodendroglial glioblastoma multiforme". Nevertheless prognosis is mostly affected by the presence of necrotic foci (23). This explains the constant reorganization through regular consensus such as the StAnne/Mayo classification and regular updates by the WHO, the latest dating from 2007; not only are classifications reshuffled, but additional entities keep being added (ref. appendix). As glioma progression is driven through acquisition of additional genetic mutations as well as epigenetic modification, it is no surprise to find that analysis of different subpopulations of GBM cells reveals not only genomic variability, but also heterogeneity at the transcriptome, microRNAome and methylome levels (14). This renders definition of the cell of origin all the more challenging. A biopsy is therefore rarely representative of the entire tumor but rather constitutes no more than a temporo-spatial snapshot of the lesion. GBM heterogeneity can be such, that one could almost assume the presence of different tumors within the same mass (24).

1.1.5 Treatment

In 1884, the first brain tumor was surgically excised by hemispherectomy (14). Currently, standard treatment of GBM includes total or subtotal surgical resection, followed by adjuvant radiation and chemotherapy. Even though it reduces the mass effect and therefore intracranial pressure, surgery alone is unfortunately not sufficient due to GBM's infiltrative nature, hence the need for adjuvant therapy (25).

Radio-chemotherapy

When employing radiotherapy, 60Gray (Gy) are usually delivered in 2Gy doses to the tumor surface with a 2-3cm margin. This option decreases irradiation of normal tissue and consequently avoids neurocognitive toxicity, leukoencephalopathy and endocrinopathies (26). Brachytherapy has shown no beneficial effect (27). Regarding chemotherapy, there is a wide range of available drugs, which we are not going to detail here. However, temolozomide has helped extend survival since its introduction in 2013 (2). Symptomatic therapies such as antalgia and anticonvulsants play an important role in GBM patient management (1).

Personalized treatment

Since GBM displays wide heterogeneity in genomic and phenotypic properties, personalized treatment may be applicable. However, in order to do so, more precise classification, leading to more specific drugs, is necessary. Subtype specific drugs showed inhibitory effects on the *in vitro* clonogenicity of patient derived cells and decreased their resistance to temolozomide (28).

Activation of *EGFR* receptor leads to downstream activation of the PI-3K/AKT pathway, which promotes cell survival, proliferation and infiltration. Blocking *EGFR* with erlotinib and gefitinib, two small molecule kinase inhibitors, have shown some temporarily promising effects, yet rapid resistance to treatment due to compensatory expression of adjacent *EGFR* receptors, such as *ERBB2* and *ERBB3* has been observed to occur. Lapatinib, another inhibitor, which blocks *EGFR* and *ERBB2*, caused reduction of AKT expression, but therapy resistance appeared over time. Erlotinib and gefitinib had some beneficial effect when *EGFRvIII* and *PTEN* are co-expressed (12).

Loss of chromosome 1p has been correlated with a positive response to procarbazine, CCNU and vincristine. When chromosome 1p/19q are lost, longer survival and response to temolozomide have been observed (13).

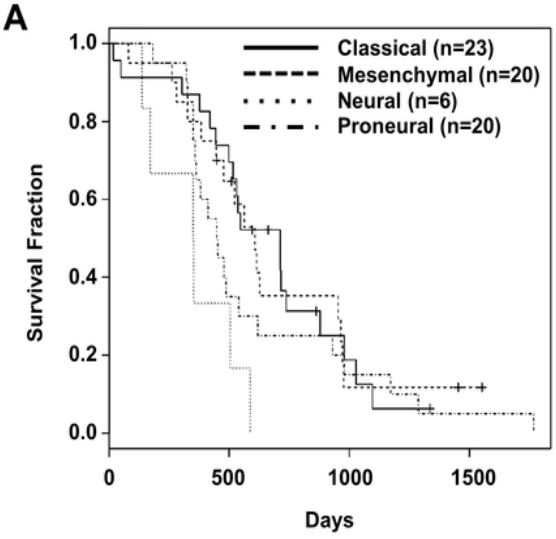
RECURRENCE

Recurrence, which is common in GBM, is believed to be driven by cancer stem cells, which show resistance to chemo and radiotherapy (29). Becavizumab, an anti-VEGF antibody, has shown clinical benefits when combined with Irinotecan in recurrent GBM (30). Curiously, although *EGFRvIII* increases proliferation and promotes anti-apoptotic signaling it is associated with better prognosis and lower recurrence. In the absence of *EGFRvIII*, the tumor appears to be resistant to temolozomide (14).

PROGNOSIS

Prognosis is affected by age, neurologic impairment, localization, radiological features (contrast enhancement), surgical resection extent, proliferation index and genetic features (23).

A predictive factor for an early response to combined radio- and chemotherapy is the presence of silencing by hypermethylation of the *MGMT* gene promoter, which does not allow alkylation of the O6 guanine and inhibits DNA repair proteins. It is associated with relatively favorable response to temolozomide therapy (12) with survival for up to 21.7months (vs. 12.7m). Hypermethylation of the MGMT promoter is found in up to 40% of GBM (19) and seems exclusive to the classical subtype (14).



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Subtype	NTP		p-value
	Median	95% CL	
Classical	713	516-979	0.0365
Mesenchymal	607	477-971	
Neural	351	171-NA	
Proneural	450	362-930	

Figure 4: Prognostic outcome of four molecular subtypes of 105 GBM patients. Reproduced from (28)

> **Prognostic factors**: EGFR(9), deletion of chromosome 1p/19q (19), IDH1 (14)
>
> **Predictive factors**: age(9), hypermethylation of MGMT (19), combination with IDH1 (14), presence of miR21 (31)

Survival rates are 42.4% at 6 months, 17.7% at one year and 3.3% at 2 years (32) with a mean age for GBM to appear at 53years (9).

1.1.6 GLIOMA CANCER STEM CELLS

As indicated previously, fatal relapse is believed to be driven by a sub-population of cells called glioma stem cells (GSC) (33), which display self-renewal properties, multi-lineage plasticity and tumor initiating capacity in xenografts generating tumors that phenocopy the tumor of origin. They are believed to occupy the top of the cell differentiation hierarchy, losing their self-renewal and tumorigenic capacities the further they advance through differentiation. They initiate tumors, are responsible for recurrence and metastasis and are more chemoradioresistant than their more differentiated progeny (34)(35). Recurrence occurs with a higher density of GSC and their presence predicts more somber prognosis. VEGF can be GSC-associated and promotes angiogenesis, hemorrhage and invasion. Also GSC are often found in vascular niches (35).

Better recognition and isolation of these cells will lead to improved patient survival by targeting therapies to GSC, sparing normal tissue. In mouse models of gliomas, specific targeting of the GSC population has been shown to prolong survival by limiting relapse rates (34). It is to be noticed that any type of therapy changes cell markers and expression profiles, making the task to identify GSC trickier after therapeutic agents have been applied (29). CD133, Sox2, Musashi and

Nestin are recognized markers for GSC identification. One of the first identified markers on cells that mimic neural stem cells is CD133. As clones undergo asymmetric division, both CD133 + and - cells are present in tumor bulks. The positive fraction is better known to be more tumorigenic, however, the negative fraction can reform tumorigenic cells and even divide again into cells carrying CD133, resulting in the idea that CD133 expression and stemness features are reversible and microenvironmentally adaptable processes (14). CD133 still remains the most satisfying marker since 60% of freshly resected GBM express it. Other identification markers have been suggested such as LCAM1, SSEA-1, A2B5, Integrin6, Nestin and Aldehyde Dehydrogenase1 but not with a reproducibly successful rate (36).

GSC can be grown and expanded as floating spheroids under serum free conditions and are commonly referred to as gliospheres. Exposure of GSC to serum triggers profound epigenetic changes leading to adhesion to substrate *in vitro*, expression of markers of differentiation, loss of clonogenicity *in vitro* and tumorigenicity *in vivo* (37).

In vivo it has been observed that GSC escape from the tumor mass to migrate to the subventricular zone after transplantation, where they find self-renewal niches. In this migration process, CXCL12 and CXCR4 are possibly implicated (38).

Despite the fact that gliospheres and adherent GBM cells share the same genetic background, their major phenotypic differences and capacity to initiate tumor growth is the result of epigenetic changes. Epigenetic mechanisms involve long non-coding RNAs (40), chromatin-based mechanism (41) and miRs, the latest **p**laying an important role in stemness as described below (42)(43). Chromatin state, with the action of transcription factors and chromatin regulators, additionally to its pre-existing state (methylation, acetylation, etc.), guides as well stemness (39).

1.2 MicroRNAs

MiRs are small non-coding RNA molecules consisting of about 22 nucleotides. In their sequence, miRs contain a seed sequence of about 2-7nucleotides long, which binds to complementary sequences primarily within the 3'UTR of targeted mRNA and leads to their degradation or translational repression, depending on the level of complementarity. Subsequently, they operate as negative regulators of protein production and are therefore crucial repressors of gene expression. As they are involved in almost every biological process – including metabolism, ageing, development - they participate toward providing homeostasis in normal tissues. However, this seemingly robust system can undergo dysregulation, one consequence of which may be transformation and tumorigenesis (44).

1.2.1 MiRs in tumorigenesis

They are several ways for miRs to be involved in tumorigenesis:

1. MiRs control expression of tumour suppressor genes. Targeting them leads to their repression and thus loss of their protective capacities. These miRs are called oncomirs;
2. Anti-oncomirs are miRs repressing oncogene mRNA, therefore they act as tumor suppressors. Any mechanism that downregulates these miRs (via silencing or deregulation in their maturation pathway) can contribute to malignancy (45);
3. Evidently, any isolated mutation in either the seed region or in the 3'UTR engenders loss of complementariy between these two regions and consequently loss of function of specific miRs;
4. MiRs go through complex processing steps any of which may be deregulated;
5. Ribonucleotide binding proteins (RBP) may bind to or in the vicinity of miR binging sites on their target transcripts, regulating their effect on target gene degradation. Changes in their expression, activity, abundance or affinity for miR binding sites may lead to transformation (46);

Generally, downregulation of all miRs has shown promotion of disease suggesting that overall tumor-suppressive function of miR is more important than its oncogenic ability. The cluster miR-17-92 is highly prevalent in a wide range of cancers and induced expression of these miRs promotes cancer (47).

1.2.2 MiRs maturation pathway

According to reviews (16)(48)(49), after being transcripted by the RNApolymerase II, primary-miRs (pri-miR) are processed into precursor-miR (pre-miR) by a Microprocessor complex; the key components of this complex consist of DROSHA, an RNA III enzyme, and DGCR8, a dsRNA-binding protein Then, pre-miRs are exported out of the nucleus by Exportin-5, which is Ran-GTP-dependent. Once in the cytoplasm, a multiprotein Dicer complex generates from the 70nt pre-miR a 21-23 mature miR. This double-stranded mature miR is then separated: one strand is loaded onto the RNA-induced silencing complex (RISC) by an Argonaut (AGO) protein, with the GW182 protein, whereas the other strand is degraded. Once attached to the complex RISC, miRs find their target transcripts and bind to their complementary sequence by their seed sequence within the 3'UTR, thereby inducing mRNA degradation or translation blockade. Sometimes a bi-functional pair of miRs, such as miR9/9*, exist where both miR strands have an inhibitory function (50). In rare cases, miRs, by binding to 5' UTR, enhance expression of different mRNAs (31). Since one miR corresponds to several complementary sequences on mRNAs, a small number of miRs can regulate a large repertoire of genes. Over 1'500 precursors and over 1900 mature *Homo sapiens* miRs have been reported in the miRBase so far (16).

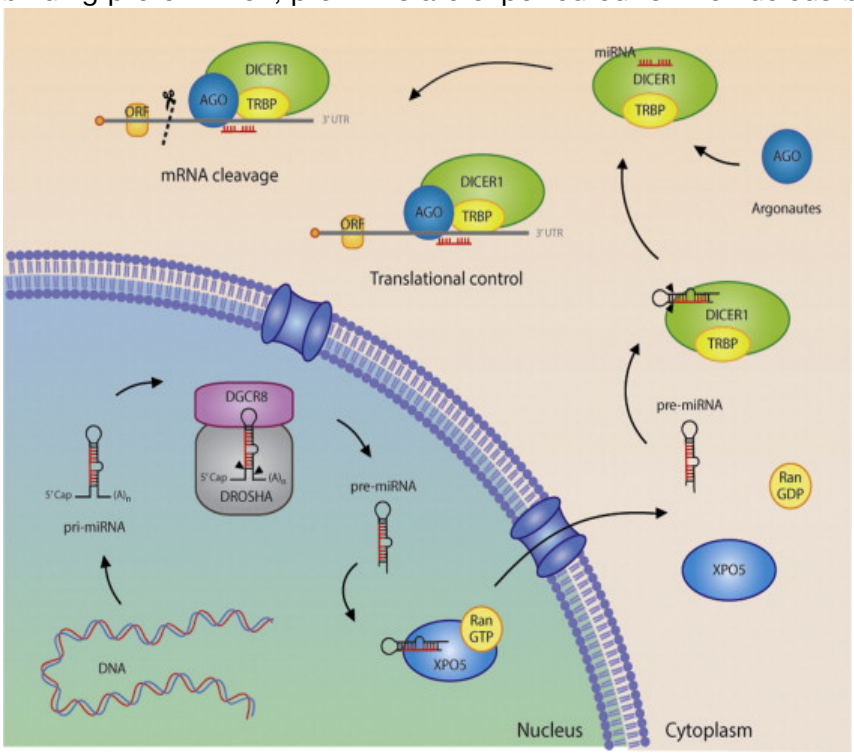


Figure 5: MiR biogenesis. Reproduced from (45)

As discussed previously, several steps in the maturation process can be dysregulated:

1. Compartmentalization has been shown to control the maturation pathway by concentrating cofactors, isolating processes in specific cellular microenvironments and therefore facilitating interaction with binding partners (51);
2. One of the crucial steps in miR biogenesis is exportation of pre-miRs outside the nucleus via Exportin 5 (XPO5), a specific karyopherin. If mutations occur in the *XPO5* gene, pre-miRs are retained in the nucleus and processing by Dicer and TRBP does not (49)occur, which has been associated with tumorigenesis. Homozygous mutations in *XPO5* are lethal events in cancer cells. It has been noticed that additional nuclear export pathway exist in C. elegans and that exportation is accelerated when DNA damage is present in an ATM-dependent manner (51)(52);
3. Stimulation of migration and metastasis happens when low Dicer processing occurs (53) (54);
4. SMAD (signal transducer of TGFβ and BMP) promotes expression of miRs by inducing the cleavage reaction by Drosha. T/B-miRs (miRs regulated via TGFβ) such as miR21 and miR199a among 20 others, contain an R-SBE domain in their stem region of their pri-miR form, which is bound by SMAD and regulated via TGFβ and BMP (55);
5. Ultimately, it has been shown that some miRs act in the nucleus by binding to promoter of specific genes and inducing their repression (51);

1.2.3 MiRs in stemness

MiRs play a crucial role in generation, maintenance and differentiation of stemness, rendering them attractive toward the development of stem cell therapies. One of the most notable examples is one of the first miR discovered: let-7. The let-7 family is involved in development and has been extensively studied in the development of *C. elegans* through sequential heterochronic gene expression, meaning that if a mutation occurs, development stops at a defined developmental stage. Due to its lack of AUG in an open reading frame, it is never transcribed, neither is it spliced due to its unsuitable size. It is complementary to the 3'UTR of heterochronic genes *lin-14, lin-28, lin-41, lin-42* and *daf-12. Let-7* and *lin-4* both regulate by their RNA *lin-14* and *lin-28* through RNA-RNA interactions via 3'UTR and so coordinate developmental timing at different stages (56). The let-7 family consists of 4 members in *C. elegans* and 15 in humans. In humans, processing of several pri-miRNAs is blocked when cells are in an embryonic state. Once time for differentiation occurs, this blockade resolves and expression occurs. Expression of mature *let-7* miR has been shown to be decreased in embryonic cells compared to differentiated cells, whereas pre-miR *let-7* remains at a constant level. This is due to *lin-28* specifically blocking the maturation process at the Microprocessor step (57). *Lin-28a* and *lin-28b* act as posttranscriptional repressors of *pre-let-7* in the cytoplasm by inducing its degradation via terminal uridylation at its 3'end and therefore blocking Dicer-mediated processing. It is not very clear whether this occurs in the Drosha step or the Dicer processing step (47). Since *let-7* is involved in differentiation, *lin-28* enables stemness maintenance and tumorigenesis. There is a double negative looping feedback between *lin-28 a*nd *let-7* (58). In summary, let-7 acts as an anti-stemness and proliferation factor, whereas lin-28 favors stemness. (59). In combination with Oct4, Nanog and Sox2, lin28 can reprogram pluripotency in fibroblasts, leading to induced pluripotent stem cell (iPSC) formation (57).

Due to their important role in stemness, it seems reasonable to propose that miRs are also involved in cancer stem cell establishment and maintenance. Establishment of tumor-associated miR expression repertoires may help develop miR-based targeted therapies aimed at rectifying miR dysregulation in GSC (16).

1.2.4 MiRs & therapy

New therapies including targeted, antiangiogenic, and cancer stem cell-specific therapies as well as specific vaccines are being developed, bringing hope to this currently incurable disease (1).

Regarding miRs, targeted therapies follow two axes: inhibition of oncogenic miRs or replacement of tumor-suppressive miRs. Replacement therapies appear to be more practically feasible, and some potentially promising results have been obtained with miR34 (60). However, progress has to be achieved on the delivery system, especially with respect to overcoming the Blood/Brain barrier. Using the non-metastatic property of GBM for local delivery might be an option. Some new insights in molecular therapies have been shed, since the discovery of intercellular communication via secreted functional miRs. This suggests that regulation of aberrant miR expression may enhance chemo- and/or radiotherapy sensitivity, as well as responsiveness to molecular-targeted therapy (61).

1.2.5 MiR21

MiR21 is involved in several processes: development; cancer; cardiovascular and pulmonary diseases; and inflammation. It is located in the TMEM49 gene and is transcribed independently due to its own promoter (62). Regarding oncogenesis, miR21 is overexpressed in 6 solid cancer types (62) where it plays an anti-apoptotic role (63). The concept of oncomiR addiction has arisen ; namely dysregulation of miR21 appears to be necessary for initiation and/or maintenance of the malignant state (62). Using microarrays and qPCR, several studies (63)(64) identified miR21 (with miR10b, miR128-1 and miR128-2) as a recurrent aberrant miR in GBM pathogenesis, present in up to 44-100% of GBM (31). Its presence is correlated with more aggressive clinical outcome and worse prognosis (65). When miR21 is suppressed, decreased cell growth, increased apoptosis, reduced invasiveness and reduced tumorigenecity (via upregulation of tumor suppressor genes such as *p53*) have been observed. Its role in invasiveness is linked to its interaction with MMP inhibitors RECK and TIMP3. When looking at targets, TGFβ, p53 and mitochondrial initiated apoptosis pathways are implicated (31). One of the direct targets is IGFBP3 which acts as a tumor suppressor gene and inhibits gliomagenesis; overexpression of IGFBP3 occurs with inhibiton of cell proliferation *in vitro* and tumor formation *in vivo* when miR21 is suppressed (66). Recent studies have shown that aberrant miR expression, such as miR21, has been correlated to radiation and chemotherapy resistance, especially temolozomide. Overexpression of miR21 inhibits TMZ-induced apoptosis via Bax/Bcl-2 (67)(68). Mir21 has also been shown to play a crucial role in radiosensitivity via the Pi3K/AKT pathway and by modulating tumor suppressor networks (69)(70). Therefore miR21 may provide an attractive target to modulate. Some groups have started measuring it in different biological fluids for correlation with clinical behavior and outcome (62). Baraniskin et al. correlated detection by qPCR of miR21 in the cerebrospinal fluid with the presence of gliomas (71)

2. AIMS

The aims of this project are to:

- Assess miR expression profile of freshly resected GBM GSC vs. tumor bulk, sorted by magnetic separation for CD133 marker expression
- Select and validate by qPCR miRs whose expression is markedly different in CD133+ vs. CD133- cell fractions
- Determine whether a subset of differentially expressed miRs are maintained *in vitro* in cells cultured as spheres or adherent monolayers that represent the GSC and tumor bulk fractions, respectively
- Modulate miR21 expression in primary GBM spheres by infection
- Evaluate the effect of miR21 overexpression on proliferation and clonogenic capacity of spheres *in vitro* ± *in vivo* by injection of control or miR modulated cells into NOD-SCID common γKO mice
- Potentially, identify and validate mRNAs targeted by miR21

2.1 EXPECTED RESULTS

Results of this study should allow us to identify miRs that are differentially expressed in GSC vs. GBM tumor bulk, either in freshly removed tumors sorted for CD133+ cell subpopulations or *in vitro* under culture conditions that maintain GSC (spheres) or induce differentiation (adherent cells). We expect to select a subset of miRs whose expression is specifically altered in the GSC fraction. The phenotype of cells overexpressing miR21 will be assessed. We expect to find differences in clonogenic, proliferation and tumor initiating capacities upon modulation of expression of miR21. Depending on the results obtained and schedule, we plan to seek mRNA transcripts specifically targeted by differentially expressed miR21 in GSC compared to their differentiated counterparts and that consequently contribute to maintaining the phenotype of this highly malignant cell population.

3. RESULTS

3.1 IDENTIFICATION OF MIRS EXPRESSED IN PRIMARY GLIOBLASTOMA CELL LINES *IN VITRO*

In order to establish the expression profile of miRs in primary GBM, we extracted RNA from three primary GBM cell lines (namely BT4, 8 and 11), which were grown as spheroids or adherent cell monolayers. Gliospheres are maintained and expanded under serum free conditions and stand as a model for GSC. Upon exposure to serum, GSC become adherent, undergo dramatic epigenetic reprogramming and lose their tumorigenic potential (37) (72). We performed a microRNA-array in order to compare the expression profile of glioma-stem-like cells vs. differentiated adherent counterparts. A scatterplot in **Figure 6** illustrates the top differentially expressed miRs in both culture conditions. MiR21, which will be of interest later, was surprisingly shown to be one of the top downregulated miRs in GSC, compared to adherent cells.

Interestingly, GSC expressed high levels of miR9, which was previously shown to be a critical regulator of neurogenesis and differentiation (73). MiR340 was recently reported to act as a tumor supressor miR in GBM (74). However, we observed that miR340 was upregulated in the GSC fraction.

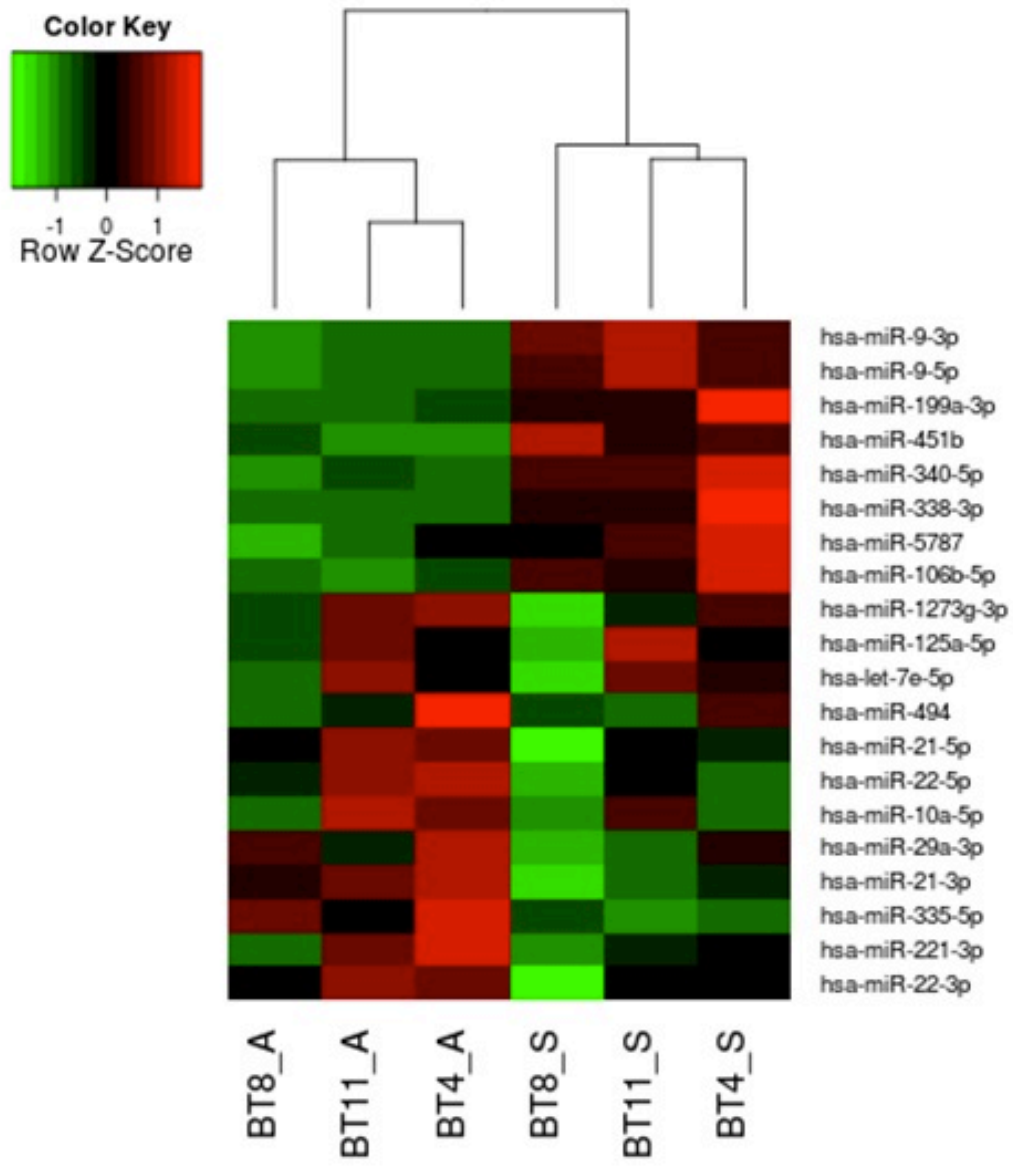


Figure 6: miRNAarray operated on RNA extracted from three GBM cell lines

BT : brain tumor, X : primary tumor number, A : adherent culture conditions, S : sphere culture conditions

To confirm the observations of the microRNAarray, we performed qPCR with specific LNA (locked nucleic acid) primers, which showed consistent results with high-troughput analysis. MiR21, miR221 and miR335 are significantly downregulated in all tumors grown as spheroids, while miR199a, miR106b and miR9 are significantly upregulated.

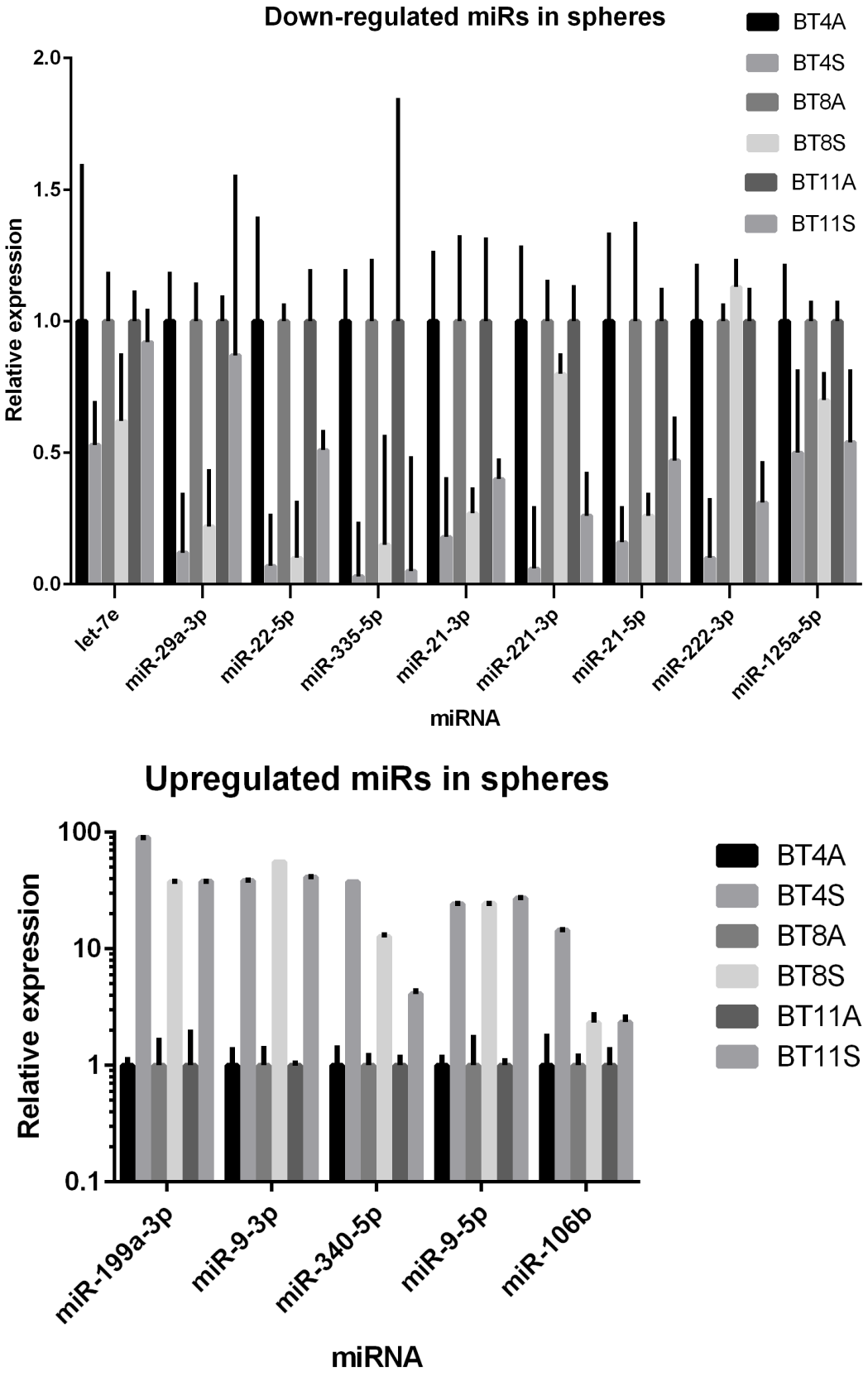


Figure 7: Validation of the miRNAarray by RealTimePCR

3.2 Extraction of CD133+ fraction in GBM and exploration of miR expression profile

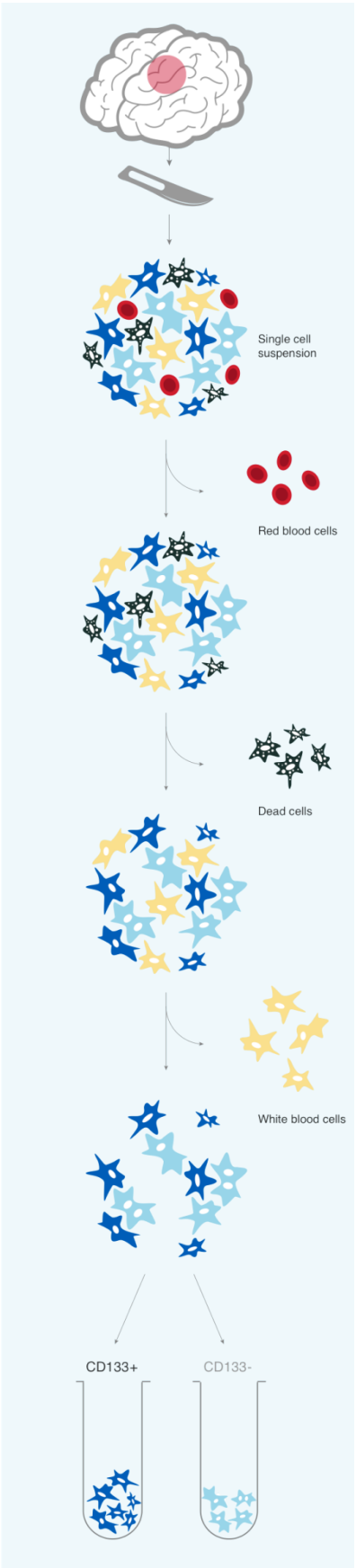


Figure 8: Essential steps in extraction of the CD133+ fraction from a freshly excised tumor

During two months, we collected freshly excised brain tumors, suspectingly GBM, based on imaging and clinical presentation, that had been operated at the CHUV. We managed to harvest three primary brain tumors during study period. Tumors were cut into small pieces, and dissociated to single cell level. Leukocytes were removed with negative CD45 selection (magnetic microbeads), while red cells were destroyed with an osmotic shock. Finally a positive selection was performed for the CD133 cell surface marker. The negative fraction was kept and named hereafter CD133 negative fraction, thereby representing the tumor bulk. This positive 133 marker, as discussed previously, represents a robust marker for the GSC fraction (72).

Unfortunately, one primary tumour appeared to be an oligodendroglioma on attentive histopathological examination and had to be excluded of analysis. Out of the two remaining tumours, one GBM did not provide a sufficient output of live cells that withstood the entire process, leaving us with only one sample to analyze.

On that specimen, a qPCR was performed in the perspective of comparing miRs expression profile between CD133+ and CD133- fractions in primary GBM and correlating it with the *in vitro* sphere-adherent culture conditions. A satisfactory concordance exists in the expression pattern between the primary tumor (CD133 positive vs. negative fraction) and the three primary cell lines grown either as spheres or adherent cells. Taken together, these results suggest that the sphere and adherent culture represent a robust model to study miRs *in vitro*, which reflects the *in vivo* expression profile of CD133+ vs. CD133- fractions, respectively. Although, it must be mentioned that these results are difficult to generalize based on a single freshly sorted tumour, and would require additional samples to confirm these observations.

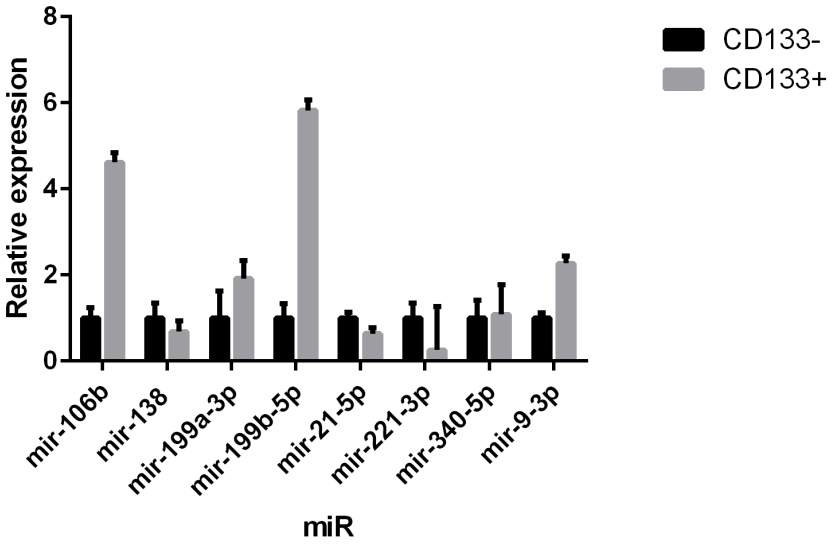


Figure 9: Validation of CD133+ vs. CD133- fraction miR expression profile

3.3 Overexpression of miR21 in GSC

Our work allowed us to identify miRs differentially expressed between distinct cellular states in GBM, both *in vivo* and *in vitro.* To address the functional relevance of these observations, we re-expressed one of the top downregulated miR in GSC. Although, miR21 was previously characterized as an oncomiR (75), the fact that it was strongly downregulated in GSC raised the question whether it may specifically silence stem-cell programs. Therefore, we performed lentiviral-mediated overexpression of miR21 in one batch of primary glioma stem cells (BT4) with miR21 precursor. MiR precursors are usually expressed rather than mature miRs, as they are more stable and subject to maturation that occurs for endogeneous miRs. This plasmid also contains a green fluorescent protein (GFP) that allows visual control of infection and a puromycin resistance gene for selection of infected cells.

3.3.1 Microscopic examinations

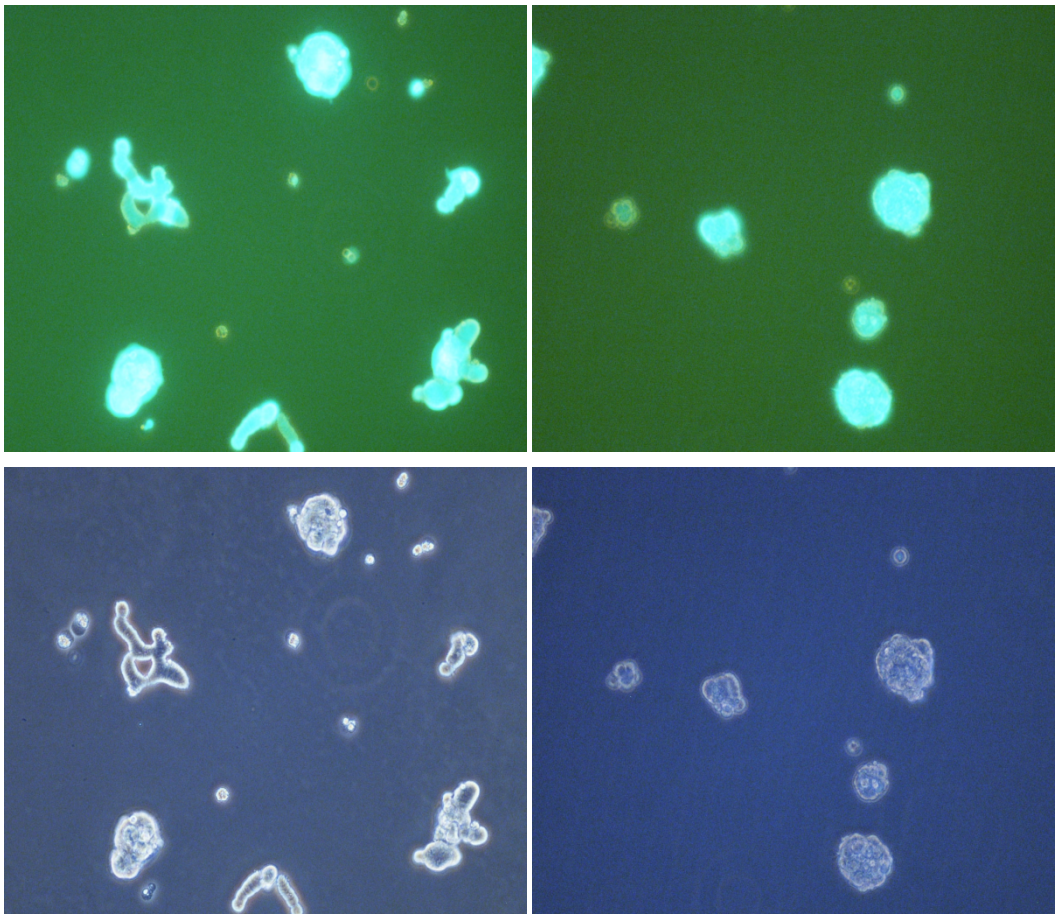
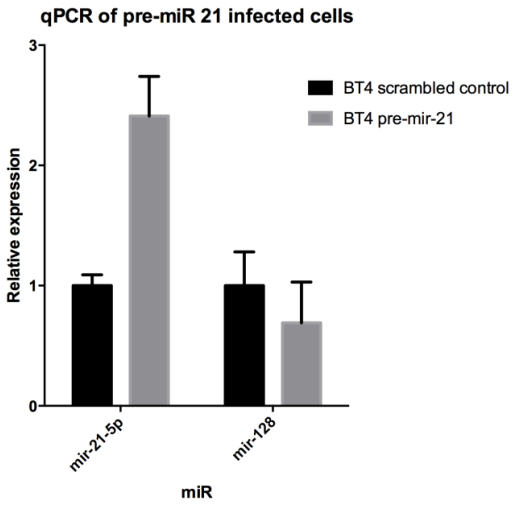


Figure 10: Gliospheres (BT4 line) infected with pre-miR21(*left)* or Scrambled-control (Scr-ctrl) *(right)* lentivirus. Upper panel : GFP fluorescence. Lower panel : white light. Pictures were taken after selection with puromycin



Infection efficacy was tested by qPCR, showing about 2.5 fold upregulation of miR21- compared to Scr-ctrl-infected cells, consequently validating the efficiency of our tools (**Figure 10**). Additionally, we tested the modification of expression of an unrelated and non-differentially expressed miR, namely miR128, to show the specificity of our infection. Moderate virus titer was used to achieve an overexpression that was in the physiological range of expression observed in adherent cells.

Figure 11: qPCR of miR21 and miR128 on BT4 spheres infected with mir21 precursor or Scr-ctrl

3.3.2 GSC OVEREXPRESSING MIR21 - CLONOGENICITY

After having confirmed efficiency of miR21 re-expression in gliospheres (BT4), we performed some functional assays comparing the overexpressing cells vs. Scr-ctrl. First, we studied the clonogenic capacity of the spheres.. A clonogenic assay is a method that evaluates the self-renewal properties of CSCs by assessment of sphere formation at low density of cell seeding. We noticed no significant change in sphere formation capacity between miR21-infected cells compared to Scr-ctrl. These results suggest that reexpression of miR21 alone is not able to impair the self-renewal properties of GSC.

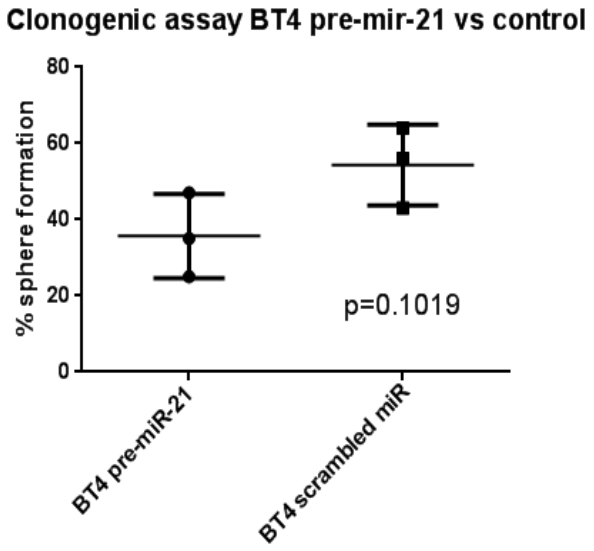


Figure 12: Clonogenic assay of gliospheres infected with miR21 vs. Scr-ctrl lentivirus

3.3.3 GSC OVEREXPRESSING MIR21 - PROLIFERATION ASSAY

We evaluated whether there was any change in proliferation rates between pre-miR21-infected cells and Scr-ctrl. A BRDU (5'bromo-2'uridine) proliferation assay revealed no significant difference between the two conditions: suggesting that miR21 is not involved in proliferation control of glioma stem cells.

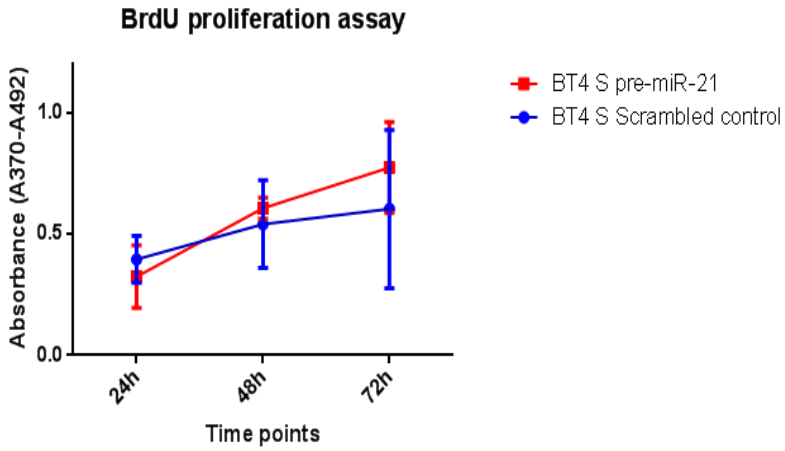


Figure 13: Proliferation assay of pre-miR21 lentivirus

4. DISCUSSION

4.1 CHARACTERISING MIR EXPRESSION PROFILES *IN VIVO* AND *IN VITRO*

Our study determined via qPCR and microRNAarray a general panel of miRs differentially expressed in GSC and their differentiated counterparts *in vitro*, whose trend was then confirmed via RealTime PCR *in vivo*. In both settings, miR21 turned out significantly downregulated in glioma stem-like cells compared to differentiated cells. All in all, these results suggest that *in vitro* the sphere and adherent culture conditions stand as a relatively genuine reflection of miRs *in vivo*, which are recognizable with CD133+ versus CD133- surface markers respectively. Obviously, additional freshly excised GBM samples are necessary to affirm this statement.

Even though we observe the same tendency in both frameworks, it should not be forgotten that we are manipulating models, which are not entirely representative of the *in vivo* complexity of GBM.

- First of all, the tumor type of origin, for either the cell lines or the *in vivo* model, has to be considered: are we facing a recurrence or a primary GBM? What kind of subtype is it (classical, mesenchymal, neural, proneural)? Has the tumor already been treated with either chemo or radiotherapy? All these factors can influence the quantity of GSC but also the features they are showing — especially regarding their expression panels. The tumour that was harvested at CHUV was a recurrence of GBM. GBM cell lines that were used join the proneural subtype. As described in the introduction, all these different expression profiles in all these settings still remain to be established ;
- Secondly, GBM has been described as heterogenous inter- (20) and intratumorally (22)(21), which, evidently, is of influence when studying only a few samples and data derived from a collection of cells;
- Moreover, either when being excised from the brain environment or having been in culture for several cycles, changes take place in cells on a transcriptome basis, but even more likely on a epigenetic basis: reprogramming may occur and miRs expression profiles may adapt to the new environment (76);
- Finally, GSC's identification is not entirely established yet. Even though, there exists a clear consensus towards CD133 surface marker as an identification tool for GSC, CD133- cells have sometimes shown stem cell properties as well (14);

Since change in GBM occurs in space and time, improvements can be done by:

- Fine-tuning subtypes characterisation, especially regarding miRs (18);
- Developing systems for multiple biopsy execution through time and space (14);
- Bettering biomarkers' specificity: via radiological imaging or repeated blood samples for circulating biomarkers (14);
- Precisely describing or finding a single, instead of a combination of identification marker for GSC (77);

In the literature, only one publication was found to study the same subject *in vitro*. It is showing similar results: downregulation of miR21, miR29a, miR221 and miR222 and upregulation of miR106b in GSC compared to differentiated GBM cells (78). It is of note that Wu et al. (79) agreed on the same system representing spheres *in vitro, a*s a CD133+ population *in vivo*, which stands as a model for GSC.

In vivo studies display concordant observations towards the following miRs: downregulation of miR21, miR9, miR340 and miR221 (79) and upregulation of miR221(80) and miR106b (80) (50) in the CD133+ fraction. Only Wu et al. (79) disagreed on miR106b, which they observed as downregulated in GSC.

All in all, as noticed, many researchers agree on performing high-throughput analysis since, as Melo et al. would describe, entire miRnome and not only a single oncogenic or tumour-suppresive miRs is disrupted in cancer (51).

4.2 FUNCTIONALITY OF MIR21

After having overexpressed miR21 via infection and selected cells for their puromycin resistance, we observed some slight phenotypic differences under the microscope. Infected cells, with either Scr-ctrl or miR21, expressed GFP, which allowed improved visualisation. Under microscopic examination, mir21-infected cells, as opposed to controls, did not 100% manage to rebuild spheres after five days and some of them remained as aggregates. Yet, the results did not show a drastic contrast. When extracting RNA from these modified cells and performing a qPCR, we confirmed the infection's efficacy and validated the differentially expressed miRs profile.

From a clonogenicity and proliferation point of view, we did not observe a statistically relevant divergence between these two populations, despite detecting a slight trend towards self-renewal decrease and proliferation enhancement in miR21-infected cells. In order to improve these results, additional sampling is necessary to reduce error bars. Seemingly, miR21 might be partially involved in tumorigenicity, however this specific miR is probably not the only one responsible for initiating tumours. Therefore, studying other miRs, in conjuncture with miR21, using a similar approach might be of interest in establishing involvements of miRs in tumorigenicity of GSC.

According to literature, miR21 has been described as one of the main oncogenes in GBM and its presence is inversely correlated to survival (66). Noticeably, this evidence is derived from datasets obtained from tumor-bulks, which do not reflect the specificities of the different cell population observed in intra-tumoral heterogeneity of GBM, letting us hypothesize that miR21 state is cell-dependent within GBM. Via the *p53* network*, TGF-beta* and mitochondrial apoptosis tumor suppressors genes, miR21 increases apoptosis and cell cycles arrest and represses tumor growth (70). When being depleted, it activates caspases, which increase apoptosis as well (63). Between GBM and normal brain tissue, its oncomiR status has been well established (75) and its level of upregulation in tumor tissue is correlated with progression of gliomas towards more aggressive grade (81). Despite being linked to glioma angiogenesis, miR21-positive tumor cells do not display stem cell features; in concordance with our results, expression of stem cell markers, such as CD133, was not correlated to miR21 (82).

Apropos of clonogenicity, no studies have been performed in regards to miR21 and GSC; only miR135b (83) and miR130b (84) have been described to affect clonogenic abilities. Referring to proliferation, miR21 has been depicted to improve proliferation *in vitro* and tumor size *in vivo* when being overexpressed in all GBM cells (66). By directly downregulating FASLG protein expression, overexpression of miR21 in GBM (and GSC, which does not match our results) has been shown to regulate GSC apoptosis and enhance GSC proliferation (85).

Altogether, seeing the amount of studies in the matter, these observations indicate that the therapeutic benefit of miR21 modulation is still debatable (62).

5. CONCLUSION AND FURTHER DEVELOPMENTS

In summary, miR21 is downregulated in cancer cells that display stem-cell traits compared to differentiated GBM cells *in vitro* as well as *in vivo*. We hypothesized that re-expression of miR21 in GSC may silence stem-cell programs and reduce their tumorigenic properties. Our *in vitro* observations failed to reveal any difference in clonogenic and proliferation capacity of miR21 overexpressing GSC.

Since miR21 is downregulated in GSC (the tumorigenic fraction of GBM), despite being an established upregulated oncomiR in the tumor bulk, this work suggests that miR21 implication in GBM development does not occur via GSC.

The following steps of this study would include enhanced description of miR21 overexpressing GSC in order to evaluate their tumor-initiating capacity, which remains one of the hallmarks of GSC. Additional functional assays such as, *in vitro*, apoptosis evaluation and *in vivo* studies by intracranial injection in NOD-SCID common γKO mice would complete the assessment. Identification of precise mRNA targets of miR21 by microArray and then qPCR for confirmation is the subsequent stage. Supplemental proteinomics studies via Western Blot and qPCR will add the final touch to the project.

Now that miR21 is starting to be established as a diagnostic and pronostic tool, this type of project will allow us to identify distinct targets for radical treatment and have a major impact on clinical outcome, particularly regarding recurrence.

6. METHODS

6.1 IDENTIFICATION OF MIRS EXPRESSED IN PRIMARY GLIOBLASTOMA CELL LINES *IN VITRO*

6.1.1 CELL CULTURE

Surgical specimens of GBM tumours were collected at Massachusetts General Hospital following approval by the institutional review board (IRB). Glioma stem cells used in this study have been thoroughly characterized in previous work (86)(87)(88). GSC (BT4, 8 & 11) were grown in Neurobasal medium (Invitrogen) supplemented with L-glutamine (Glutamax, Gibco), B27 supplement (Invitrogen), N2 supplement (Invitrogen), antibiotics (PenStrep 1%), 20 ng/mL recombinant human EGF (R&D Systems), 20 ng/ml recombinant human FGF2 (R&D Systems). Spheres were mechanically dissociated and diluted once they reached a diameter of 100 um. At each passage a fraction of the cells was frozen to maintain a low passage source of cells for experimental procedures (<25). GSC differentiation was induced by addition of 10% FCS (+ NEAA) and withdrawal of growth factors for 7 days on 100ug/mL poly-D-lysine (ref. P7280, Sigma) and 15ug/mL laminin (ref. L2020, Sigma) coated plates. Cells were subsequently grown and expanded as adherent monolayers.

6.1.2 RNA EXTRACTION, CDNA SYNTHESIS AND QUANTITATVE PCR

RNA extraction was performed using miRCURY RNA isolation kit (Exiqon) in conjunction with DNAse treatment (Qiagen). 500ng of RNA were reverse-transcribed using M-MLV (Promega) to generate cDNA. qRT-PCR was performed with Power SYBR Green mastermix (Applied Biosystems). Each PCR reaction was performed in triplicate and normalized to endogeneous controls PPIA and 18S (ThermoFisherScientific). For microRNA-qRT-PCR, 50ng of template RNA were retro-transcribed using a universal cDNA synthesis kit (Exiqon). qRT-PCR was done in triplicate with primers annealing to mature miR (microRNA LNA PCR primer sets, Exiqon) and relative quantification of each target was normalized to RNU5G and SNORD49a (Exiqon).

6.1.3 miRNA array

Probe intensities provided by the facility were analyzed using limma. Log-transformed values were normalized between arrays using cyclic-loess. For hierarchical-clustering, the 20 miRs with highest fold-changes were clustered by complete-linkage using base R functions.

6.2 Extraction of CD133+ fraction in GBM and exploration of miR expression profile

6.2.1 CD133 extraction

Surgical specimens were harvested at the CHUV Hospital, Lausanne. Among the three primary tumors collected, only one sample had matter for analysis. MACS Miltenyi Biotec kits were used to first dissociate brain tumors with papaïne, remove dead cells and white blood cells (using CD45 microbeads for WBCs) and finally seperate CD133 + and – fractions for analysis. Red blood cells were osmotically exploded applying ACK after the dissociation stage. Every step was thoroughly done according to the manufacturers instructions. After GSC selection, RNA extraction and qPCR were performed as described above.

6.3 Overexpression of miR21 in GSC

6.3.1 Lentiviral infections, overexpression plasmids

Infection of primary GBMs was performed using lentiviruses. For viral production, HEK 293T packaging cells were transfected with the plasmid of interest, PMD2G and PCMVdR8 using FuGene HD (Promega). Virus containing media was harvested after 72 hours, 0.45um filtered (Millipore) and ultracentrifuged for 2 hours at 4°C, at 19'500 RPM using a SW28 rotor (Beckman Coulter). Supernatants were decanted and pellets were re-suspended by gentle pipetting. GBM cells were infected for 16 hours and selection was added 48 hours later for four days (Puromycin, 2mg/mL). Cells were immediately used for experiments. For overexpression experiments, miR21 was amplified using plasmids incoding pre-miR21 sequences (controlled by a *CMV* promoter), puromycin resistance cassette and GFP for luminescent control. The plasmid's sequence is verified and manufactured by BioCAT Laboratories. Plasmid purification was achieved using JetStar kit (Genomed) according to the manufacturers procedure.

6.3.2 GSC overexpressing miR21 - Clonogenicity

Glioma stem cells infected with Scr-ctrl or miR21 were mechanically dissociated and plated at single cell density in 96-well low adherence plates. Sphere number was assessed four weeks later by imaging. Each clonogenic assay was performed in triplicate.

6.3.3 GSC overexpressing miR21 - Proliferation assay

Cell Proliferation ELISA (BrdU) protocols by Roche were applied to establish proliferation. Glioma stem cells infected with Scr-ctrl vs. miR21 were spread, after careful dissociation, by 25'000 cells per wale in 5 replicates per plate in four 96-wales plates (not including controls). Each 12h, one of the 96-wales plate had BRDU (5-bromo-2'-deoxyuridine) added to cells at a 1:1'000 concentration. After overnight incubation, and supernatant elimination, each plate was heated at 60°C for 1h and then stored at 4°C in aluminium in the wait of the other plates to proliferate until the desired time. Revelation was unveiled according to the manufacturers recommendations (using FixDenat and anti-BrdU solutions) and absorbance was measured a 492 nm with a luminometer.

APPENDIX

Figure 14: The 2007 WHO Classification of Tumours of the Central Nervous System. Reproduced from (23)

TUMOURS OF NEUROEPITHELIAL TISSUE

Astrocytic tumours	
Pilocytic astrocytoma	9421/1[1]
Pilomyxoid astrocytoma	*9425/3**
Subependymal giant cell astrocytoma	9384/1
Pleomorphic xanthoastrocytoma	9424/3
Diffuse astrocytoma	9400/3
Fibrillary astrocytoma	9420/3
Gemistocytic astrocytoma	9411/3
Protoplasmic astrocytoma	9410/3
Anaplastic astrocytoma	9401/3
Glioblastoma	9440/3
Giant cell glioblastoma	9441/3
Gliosarcoma	9442/3
Gliomatosis cerebri	9381/3
Oligodendroglial tumours	
Oligodendroglioma	9450/3
Anaplastic oligodendroglioma	9451/3
Oligoastrocytic tumours	
Oligoastrocytoma	9382/3
Anaplastic oligoastrocytoma	9382/3
Ependymal tumours	
Subependymoma	9383/1
Myxopapillary ependymoma	9394/1
Ependymoma	9391/3
Cellular	9391/3
Papillary	9393/3
Clear cell	9391/3
Tanycytic	9391/3
Anaplastic ependymoma	9392/3
Choroid plexus tumours	
Choroid plexus papilloma	9390/0
Atypical choroid plexus papilloma	*9390/1**
Choroid plexus carcinoma	9390/3
Other neuroepithelial tumours	
Astroblastoma	9430/3
Chordoid glioma of the third ventricle	9444/1
Angiocentric glioma	*9431/1**

[1] Morphology code of the International Classification of Diseases for Oncology (ICD-O) (614A) and the Systematized Nomenclature of Medicine (http://snomed.org). Behaviour is coded /0 for benign tumours, /3 for malignant tumours and /1 for borderline or uncertain behaviour.

* The italicised numbers are provisional codes proposed for the 4th edition of ICD-O. While they are expected to be incorporated into the next ICD-O edition, they currently remain subject to change.

Neuronal and mixed neuronal-glial tumours	
Dysplastic gangliocytoma of cerebellum (Lhermitte-Duclos)	9493/0
Desmoplastic infantile astrocytoma/ ganglioglioma	9412/1
Dysembryoplastic neuroepithelial tumour	9413/0
Gangliocytoma	9492/0
Ganglioglioma	9505/1
Anaplastic ganglioglioma	9505/3
Central neurocytoma	9506/1
Extraventricular neurocytoma	*9506/1**
Cerebellar liponeurocytoma	*9506/1**
Papillary glioneuronal tumour	*9509/1**
Rosette-forming glioneuronal tumour of the fourth ventricle	*9509/1**
Paraganglioma	8680/1
Tumours of the pineal region	
Pineocytoma	9361/1
Pineal parenchymal tumour of intermediate differentiation	9362/3
Pineoblastoma	9362/3
Papillary tumour of the pineal region	*9395/3**
Embryonal tumours	
Medulloblastoma	9470/3
Desmoplastic/nodular medulloblastoma	9471/3
Medulloblastoma with extensive nodularity	*9471/3**
Anaplastic medulloblastoma	*9474/3**
Large cell medulloblastoma	9474/3
CNS primitive neuroectodermal tumour	9473/3
CNS Neuroblastoma	9500/3
CNS Ganglioneuroblastoma	9490/3
Medulloepithelioma	9501/3
Ependymoblastoma	9392/3
Atypical teratoid / rhabdoid tumour	9508/3

TUMOURS OF CRANIAL AND PARASPINAL NERVES

Schwannoma (neurilemoma, neurinoma)	9560/0
Cellular	9560/0
Plexiform	9560/0
Melanotic	9560/0
Neurofibroma	9540/0
Plexiform	9550/0
Perineurioma	
Perineurioma, NOS	9571/0
Malignant perineurioma	9571/3
Malignant peripheral nerve sheath tumour (MPNST)	
Epithelioid MPNST	9540/3
MPNST with mesenchymal differentiation	9540/3
Melanotic MPNST	9540/3
MPNST with glandular differentiation	9540/3

TUMOURS OF THE MENINGES

Tumours of meningothelial cells	
Meningioma	9530/0
Meningothelial	9531/0
Fibrous (fibroblastic)	9532/0
Transitional (mixed)	9537/0
Psammomatous	9533/0
Angiomatous	9534/0
Microcystic	9530/0
Secretory	9530/0
Lymphoplasmacyte-rich	9530/0
Metaplastic	9530/0
Chordoid	9538/1
Clear cell	9538/1
Atypical	9539/1
Papillary	9538/3
Rhabdoid	9538/3
Anaplastic (malignant)	9530/3
Mesenchymal tumours	
Lipoma	8850/0
Angiolipoma	8861/0
Hibernoma	8880/0
Liposarcoma	8850/3
Solitary fibrous tumour	8815/0
Fibrosarcoma	8810/3
Malignant fibrous histiocytoma	8830/3
Leiomyoma	8890/0
Leiomyosarcoma	8890/3
Rhabdomyoma	8900/0
Rhabdomyosarcoma	8900/3
Chondroma	9220/0
Chondrosarcoma	9220/3
Osteoma	9180/0
Osteosarcoma	9180/3
Osteochondroma	9210/0
Haemangioma	9120/0
Epithelioid haemangioendothelioma	9133/1
Haemangiopericytoma	9150/1
Anaplastic haemangiopericytoma	9150/3
Angiosarcoma	9120/3
Kaposi sarcoma	9140/3
Ewing sarcoma - PNET	9364/3
Primary melanocytic lesions	
Diffuse melanocytosis	8728/0
Melanocytoma	8728/1
Malignant melanoma	8720/3
Meningeal melanomatosis	8728/3
Other neoplasms related to the meninges	
Haemangioblastoma	9161/1

LYMPHOMAS AND HAEMATOPOIETIC NEOPLASMS

Malignant lymphomas	9590/3
Plasmacytoma	9731/3
Granulocytic sarcoma	9930/3

GERM CELL TUMOURS

Germinoma	9064/3
Embryonal carcinoma	9070/3
Yolk sac tumour	9071/3
Choriocarcinoma	9100/3
Teratoma	9080/1
Mature	9080/0
Immature	9080/3
Teratoma with malignant transformation	9084/3
Mixed germ cell tumour	9085/3

TUMOURS OF THE SELLAR REGION

Craniopharyngioma	9350/1
Adamantinomatous	9351/1
Papillary	9352/1
Granular cell tumour	9582/0
Pituicytoma	*9432/1**
Spindle cell oncocytoma of the adenohypophysis	*8291/0**

METASTATIC TUMOURS

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