Alma Mater Studiorum • Università di Bologna

FACOLTÀ DI SCIENZE MATEMATICHE, FISICHE E NATURALI

Dottorato di Ricerca in

Biologia cellulare, molecolare e industriale -

Progetto n. 1: Biologia e Fisiologia cellulare

Ciclo XXV

Settore Concorsuale di afferenza: 06/A2
Settore Scientifico disciplinare di afferenza: MED/04

MicroRNAs expression analysis in high grade gliomas

Presentata da

Michela Visani

Coordinatore Dottorato
Prof.ssa Michela Rugolo

Relatore
Prof.ssa Annalisa Pession

Esame finale anno 2013

Abstract

Several biomarkers had been proposed as useful parameters to better define the prognosis or to delineate new target therapy strategies for glioblastoma (GBM) patients. MicroRNAs could represent interesting molecules, for their role in tumorigenesis and cancer progression and for their specific tissue expression. Although many studies have tried to identify a specific microRNAs signature for glioblastoma, by now an exhaustive GBM microRNAs profile is far to be well defined.

In this work we set up a real-time qPCR, based on LNA primers, to investigate the expression of 19 microRNAs in brain tumors, focusing our attention on GBMs.

MiRNAs expression in 30 GBM paired FFPE-Fresh/Frozen samples was firstly analyzed. The good correlation obtained comparing miRNAs results confirmed the feasibility of performing miRNAs analysis starting from FFPE tissues. This leads to many advantages, as a good disposal of archival tumor and normal brain specimens and the possibility to verify the percentage of tumor cells in the analyzed sample. In the second part we compared 3 non-neoplastic brain references to use as control in miRNAs analysis. Normal adjacent the tumor, epileptic specimens and a commercial total RNA were analyzed for miRNAs expression and results showed that different non-neoplastic controls could lead to important discrepancies in GBM miRNAs profiles.

Analyzing 50 FFPE GBMs using all 3 non-neoplastic references, we defined a putative GBM miRNAs signature: mir-10b, miR-21 and miR-27a resulted upregulated, while miR-7, miR-9, miR-26a, miR-31, miR-101, miR-137, miR-222 and miR-330 were downregulated.

Comparing miRNAs expression among GBM group and gliomas of grade I, II and III, we obtained 3 miRNAs (miR-10b, mir-34a and miR-101) showing a different regulation status between high grade and low grade gliomas. Intriguingly, miR-10b was upregulated in high grade and significantly downregulated in low grade gliomas, suggesting that could be a candidate for a GBM target therapy.

Table of contents

Chapter 1:

Introduction

1.1 Gliomas: from classification to target treatments

Malignant gliomas constitute the 80% of primary malignant brain neoplasia and, represent the most common lesions among all primary brain tumors, covering the 30% of cases with a yearly incidence of 6 cases per 100,000[1].

Gliomas could arise from de-differentiated mature neural cells, neural stem cells or from progenitor cells [2, 3]. They are usually classified depending on histopatological histotype in astrocytomas, mainly composed by fibrillary or gemistocytic neoplastic astrocytes, oligodendrogliomas, composed by monomorphic cells with uniform round nuclei; mixed oligoastrocytomas, characterized by neoplastic glial cells with astrocytic or oligodendroglial phenotypes and ependymomas, mainly constitued by ependymal cells (Table 1.1). Only astrocytic tumors represent about the 75% of all gliomas, among them glioblastoma (grade IV) account for the 54% (Table 1.1) [1].

Gliomas are further divided in low grade (grade I and II) and high grade (grade III and IV) tumors depending on grade of malignancy (Table 1.2) [4].

1.1.1 Low grade gliomas

Low grade tumors are benign lesions, circumscribed to the brain, characterized by well differentiated cells and good prognosis. They are generally solved with the only surgery: although commonly the size of the lesions is relatively small, with indolent and slow growth, when the tumor arises in a critical area of the brain it could cause severe problems if not removed. In cases where the surgical removal is not possible or not decisive, radio- and chemo-therapy are also considered. Grade I gliomas are classified as follows: subependymal giant cell astrocytoma, pilocytic astrocytoma, as astrocytic lesions, and subependymoma and myxopapillary ependymoma, as ependymal tumors (Table 1.2) [3, 4].

Grade II gliomas account for about 25% of diffuse gliomas and they are still considered relative benign tumors: they are classified in pilomyxoid astrocytoma, diffuse astrocytoma and pleomorphic xanthoastrocytoma, for astrocytic lesions, oligodendroglioma, oligoastrocytoma and ependymoma (Table 1.2) [3, 4].

Histopatological histotype	Frequency
Glioblastoma	54.0%
Anaplastic astrocytoma	5,9%
Diffuse astrocytoma	9,5%
Pilocytic astrocytoma	5,1%
Oligodendroglioma	6,2%
Oligoastrocytoma	3,3%
Ependymoma	6,7%
Other gliomas	9,3%

Table 1.1. Distribution of gliomas by histology subtypes. Only astrocytomas account for the 75% of all gliomas. Data from Dolecek et al. [1].

All of them are characterized by well differentiated cells, moderate cell density and occasionally anaplastic cells with nuclear atypia could be present [3].

They present a propensity to infiltrate the surrounding brain parenchyma that lead surgery not always possible or decisive.

In about the 70% of cases, a grade II glioma could progress to a lesion of higher grade of malignancy, related to the accumulation of genetic alterations [5].

As regards the prognosis, it is reported that a patient affected by a WHO grade II glioma typically survive more than 5 years (6-8ys for grade II astrocytomas, 12ys for grade II oligodendrogliomas, 6ys for mixed oligodendroastrocytomas) [3, 4].

Histopatological histotype / WHO grading		Astrocytomas	Oligodendrogliomas	Oligoastrocytomas	Ependymomas
Low grade gliomas (LGG)	I	Pilocytic astrocytoma Subependymal giant cell astrocytoma			Subependymoma Myxopapillary ependymoma
	II	Pilomyxoid astrocytoma Diffuse astrocytoma Pleomorphic xanthoastrocytoma	Oligodendroglioma	Oligoastrocytoma	Ependymoma
High grade gliomas (HGG)	III	Anaplastic astrocytoma	Anaplastic Oligodendroglioma	Anaplastic Oligoastrocytoma	Anaplastic ependymoma
	IV	Glioblastoma Giant cell gioblastoma Gliosarcoma		Glioblastoma with oligodendroglial component (GBMO)	

Table 1.2. Classification of gliomas depending on histopatological histotype and grade of malignancy.

1.1.2 High grade gliomas

Grade III gliomas constituted about the 25% of diffuse gliomas and they could present as primary lesions or secondary tumors derived from a grade II glioma, sharing the same histopathological phenotype [3]. Anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma, and anaplastic ependymoma are grade III gliomas (Table 1.2), all of them are characterized by high proliferation rate, invasiveness and poor prognosis [3, 4].

A regional or diffuse analpasia is present, cell density increases in comparison with a grade II, more nuclear atypias and mitotic activity are described.

These anaplastic lesions are generally treated with conventional surgery, radio- and chemo-therapy. The median survival is about 3 years for anaplastic astrocytomas and mixed anaplastic oligoastrocytomas, while the oligodendroglial subtype has a better prognosis (3-10 years) [3, 4].

Glioblastoma, giant cell glioblastoma, gliosarcoma and glioblastoma with oligodendroglial component are grade IV astrocytic gliomas, except for the latter one which present a mixed histotype (Table 1.2) [4]. Glioblastoma (GBM) is the most malignant and the most frequent brain tumor of the adult, accounting for about 50% of diffuse gliomas [3]. It is constituted by pleomorphic astrocytic tumor cells and it is characterized by poor cell differentiation, high cell density, marked nuclear atypia, high mitotic activity. Moreover, marked microvascular

proliferation and necrosis are peculiar features, in association with a high proliferation rate, marked invasiveness and resistance to chemo- and radio-therapies [3]. Glioblastoma could arise without an evidence of a precursor lower grade lesion (primary glioblastoma, which represent about the 90% of cases in older patients) or could progress from a grade II astrocytoma (secondary glioblastoma, about the 10% of cases with an average onset under 45ys) [3, 5]. Primary and secondary GBMs are indistinguishable from a histopathological point of view, but they are characterized by different molecular alterations (see paragraph 1.1.3 and Figure 1.2) [5, 6]. Depending on pathway alterations and gene expression profile, primary GBMs are further classified in four transcriptional subclasses: classic, neural, proneural, and mesenchymal [6, 7].
Despite progresses in surgical techniques, radio-, chemo- and target therapies, the GBM prognosis remains poor, with a median survival less than one year [4, 8-10]. An early onset, prompt diagnosis, feasible surgical resection, adjuvant treatments, giant-cell or oligodendroglial subtypes are associated to a better prognosis [10].

1.1.3 Molecular classification of gliomas

A classification based on grading and histological features is not exhaustive to deeply characterize gliomas.
In the last years several prognostic and predictive biomarkers were proposed, attending to explain the great variability in biologic behavior which characterizes each diagnostic category. For glioblastomas, for which the conventional therapies are not enough to contrast tumor progression and to ensure a better prognosis, the discovery of new genetic alterations could be useful to define potential molecular targets for developing specific treatments.
Taking in consideration the classical classification of gliomas, they were further characterized according to their molecular alterations, often associated to specific histological subtype. The most common alterations described in low and high grade gliomas are listed below (when known, it is reported also the prognostic value of a particular alteration):
- *IDH1* mutations. Heterozygous *IDH1* (isocitrate dehydrogenase 1) mutations (the most common one is the R132H substitution) are prognostic factors

associated to younger onset and longer overall survival. They are detected in grade II and III gliomas and in secondary glioblastomas (80%). It was also found in <10% of primary GBMs (Figures 1.1 and 1.2) [2, 3, 5, 6, 10].

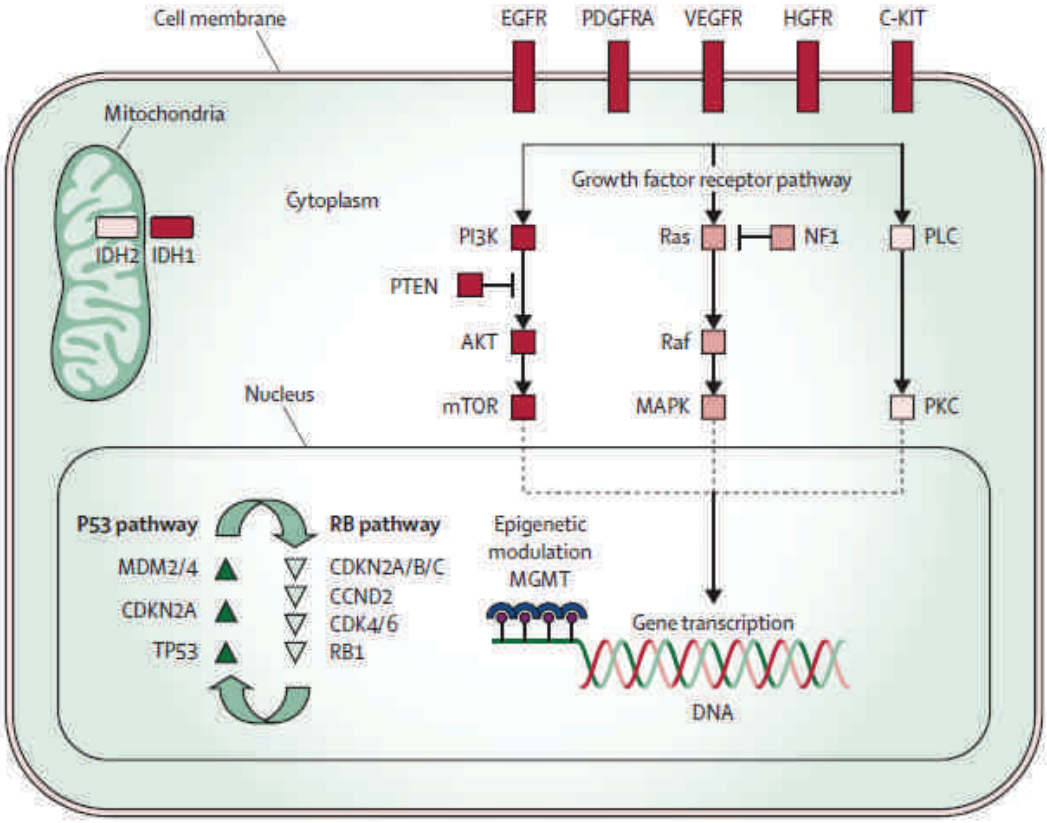


Figure 1.1. Main molecular pathways involved in glioma biology. IDH1/2: isocitrate dehydrogenase 1/2. MDM2/4: mdm2/4 p53 binding protein homolog . CDKN2A: cyclin-dependent kinase inhibitor 2A. TP53: tumor protein p53. CDKN2A/B/C: cyclin-dependent kinase inhibitor 2A/B/C. RB1: retinoblastoma 1. CDK4/6: cyclin-dependent kinase 4/6. CCND2: cyclin D2. PI3K: phosphoinositide-3-kinase. PTEN: phosphatase and tensin homolog. AKT: V-akt murine thymoma viral oncogene homolog 1. mTOR: mechanistic target of rapamycin. RAS: rat sarcoma viral oncogene homolog. NF1: neurofi bromin 1. RAF: V-raf murine sarcoma viral oncogene homolog. MAPK: mitogen-activated protein kinase. PLC: phospholipase C. PKC: protein kinase C. EGFR: epidermal growth factor receptor. PDGFRα: platelet-derived growth factor receptor α. VEGFR: vascular endothelial growth factor receptor. HGFR: hepatocyte growth factor receptor. C-KIT: v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog. MGMT: O6-methylguanine-DNA methyltransferase. Figure from Ricard *et al.* [3].

- 1p/19q loss. This alteration is characteristic of gliomas with an oligodendroglial component: it is detected in up to 90% of grade II oligodendrogliomas, in 50-70%

of anaplastic oligodendrogliomas, in 30-50% of mixed oligoastrocytomas and 20-30% of anaplastic oligoastrocytomas. It is recognized as a putative prognostic and predictive value for anaplastic lesions as associated to a better prognosis, slower progression of disease and better response to treatments [2, 3, 5, 6, 10].

- p53 pathway alterations. *TP53* tumor suppressor gene is mutated or deleted in >50% of astrocytic lesions, both low grade and high grade gliomas. It is reported that p53 pathways is altered in about 87% of GBMs. *TP53* alterations are reported as mutually exclusive with amplifications of *MDM2* (10% of primary GBMs) or *MDM4* (4% of primary GBMs) that lead to inactivation of p53 cascade (Figures 1.1 and 1.2) [2, 3, 5, 6, 10].

- Rb pathway alterations. The retinoblastoma (Rb) pathway is a key regulator of cell cycle through G1 checkpoint. Alterations in this pathways are encountered in about 78% of GBMs. In particular the inactivation of *RB* gene is found in about 25% of high grade astrocytomas. In high grade gliomas functional inactivation of RB cascade is also caused by *CDK4/6* amplifications or by the deletion of *CDKN2A* gene. This latter is common in primary GBMs (50-70%): two proteins, p16/Ink4a and p14/ARF, are encoded by *CDKN2A*, leading to inactivation of Rb and p53 pathways respectively (Figures 1.1 and 1.2) [2, 3, 5, 6, 10]. By now the prognostic implication of altered p53 or Rb pathways in gliomas remain marginal and not fully understood [10].

- RTKs pathway alterations. Activations of growth factor tyrosine kinase receptors (RTKs), as EGFR, PDGFR or MET, by mutations or amplifications are described in about 70% of primary GBMs and have negative prognostic values. Moreover multiple RTKs could be found altered at the same time (especially in primary GBMs) preventing the inhibitory effect of the target inactivation of a single RTK (e.g. using Erlotinib versus EGFR).

EGFR results amplified in about 50% of GBMs and generally its amplification is associated with the expression of the truncated variant EGFRvIII (a constitutive oncogenic form due to the genomic deletion of exons 2-7). These alterations are mainly associated with high grade gliomas and represent poor prognostic factors.

PDGFR amplification/mutation or PDGF overexpression are described both in low grade gliomas and in GBMs. Elevated levels of PDGFB are described in

about the 30% of cases, often in association with *EGFR* or *MET* amplifications (Figures 1.1 and 1.2) [2, 3, 5, 6, 10].

- PI3K-Akt pathway alterations Downstream RTKs, the activation of PI3K (phosphoinositide-3-kinase) pathway is associated to a higher tumor grade, resistance to apoptosis and poor prognosis in gliomas. In response to PI3K activation, several signaling proteins are recruited, like PDK1 and Akt/PKB which activate directly or indirectly other significant substrates. For example, Akt indirectly activates mTOR (TORC2 complex) which acts promoting cell growth. In about 15% of GBMs *PIK3CA* present a gain-of-function mutation, while in about 50% of GBMs *PTEN* (phosphatase and tensin homologue), a negative regulator of PI3K-Akt-mTOR pathway, is inactivated by mutations or deletions (LOH chromosome 10). In other cases an overexpression of Akt could be detected in GBMs, in association to poor prognosis (Figures 1.1 and 1.2) [2, 3, 5, 6, 10, 11].

- Ras-MAPK signaling alterations. RTKs activate Ras-MAPK cascade: the dimerization and cross-phosphorilation of RTKs lead to the formation of binding sites for Grb2/SOS complex which turns Ras to the active form. High levels of the activated Ras-GTP, which activates MEK/ERK/transcription factors cascade, are described in high grade gliomas, suggesting that activated RTKs mitogenic/proliferative effects in GBMs are mediated also by Ras-MAPK axis. Moreover an inactivation of NF1 (neurofibromin 1), a negative regulator of Ras-MAPK cascade, is reported in about 18% of GBM cases. MAPK cascade activation is associated with increased resistance to radiotherapy (Figures 1.1 and 1.2) [2, 3, 5, 6, 10].

- MGMT promoter methylation. O6-methylguanine-DNA methyltransferase, a DNA repair enzyme, is epigenetically silenced in 40% of primary GBMs and in about 70% of secondary GBMs. This methylation status is also observed in anaplastic gliomas (50-80% of cases). *MGMT* promoter methylation is an important prognostic factors because is directly linked to chemotherapy response to temozolomide treatment. It is associated with improved outcome in patients affected by anaplastic astocytomas and oligoastrocytomas treated with

temozolomide, while in anaplastic oligodendroglioma is a prognostic but not predictive factor (Figure 1.1) [10].

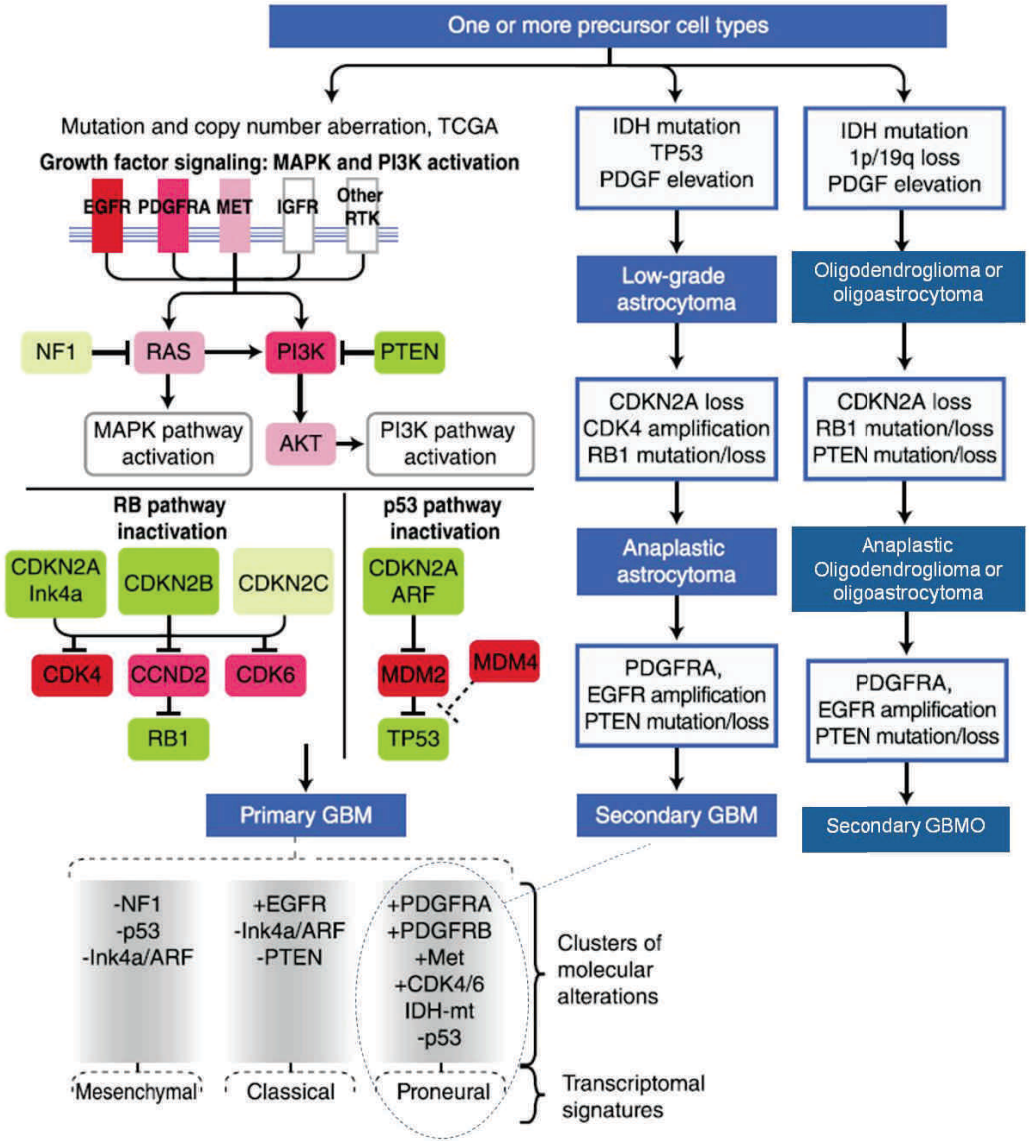


Figure 1.2 (previuos page). Molecular classification of gliomas. Figure obtained and modified from Brennan *et al.* [6]. On the left the known alterations involved in glioblastoma (GBM) *de novo* pathogenesis. Following Brennan *et al.* color scheme [6], red boxes indicate activating mutations and/or amplifications; green boxes represent silencing mutations and/or gene deletions. Darker colors represent a higher prevalence: EGFR alteration is found in approximately 50% of GBM, *CDKN2A* deletion in 50%-70%. Primary GBMs are further subclassified depending on transcriptomal patterns: *Mesenchymal* signature associated with loss of NF1 and p53 mutations; *Classical* characterized by EGFR amplification and loss of PTEN and CDKN2A; *Proneural* signature associated with PDGFR activation, IDH mutation, and amplification of CDK4 and Met. This latter one is the only associated also to secondary lesions. The fourth transcriptomal subclass, *Neural*, is not associated with a particular genotype and is not shown.
On the right, secondary lesions cascades are represented. Gliomas with oligodendroglial component are mainly characterized by 1p/19q loss and *IDH* mutations, while astrocytomas by TP53 inactivations. As indicated in the scheme and further explained in the text, the accumulation of molecular alterations lead to the secondary GBMs.

1.1.4 Further glioblastoma characterization

In addition to previous described features, Verhaak *et al.* [7] suggested a further classification of GBMs in four transcriptional subclasses according to transcriptional data: proneural, mesenchymal, classical and neural. The classical form seems to be associated with *EGFR* amplification, p16 deletion and *PTEN* loss. Mesenchymal subclass is linked to *NF1*, p53 and *CDKN2A* alterations. Proneural GBMs, with an apparent prognostic advantage in comparison to the other subtypes and the only associated to secondary lesions, reported *PDGFR*, *CDK4/6, MET* amplifications in association to *IDH1* and *PI3K* mutations and inactivation of p53 pathway (Figure 1.2). The fourth class, neural GBMs, showed a different transcriptional signature without any particular alteration [6, 7].

In addition to the previous discussed GBMs markers, other peculiar GBMs molecular alterations had been investigated. Distinctive features of GBMs in comparison to a lower grade glioma are the presence of marked necrosis, vascularization, tumor cell invasion and resistance to apoptosis. Previous studied focused their attention on these features to identify other putative biomarkers and therapeutic strategies.

Recently the role of the protein Bcl2L12, an inhibitor of mitochondrial apoptosis pathway overexpressed in primary GBMs, was studied. The function of this

protein is reported to be relevant to the necrotic process: the suppression of caspase activity in mitochondrial death signaling redirects the death program from apoptosis to necrosis [5, 12, 13].

The marked microvascular proliferation in primary and secondary GBMs is mainly driven by the overexpression of several pro-angiogenic factors in response to intratumour hypoxia: matrix metalloprotease (MMP-2), angiopoietin-1, phosphoglycerate kinase (PGK), erythropoietin (EPO), and vascular endothelial growth factor VEGFA [14].

At the base of GBM cells invasiveness, several genes play a crucial role, as a metalloproteinases family members (e.g. MMP2 and MMP9 correlate with invasiveness, proliferation and prognosis in high astrocytomas) or their inhibitor proteins (TIMPs). Moreover also urokinase-type plasminogen activator (uPA) and αVβ3 integrin complexes are overexpressed in GBMs and they are relevant to GBM invasion [5].

Finally another characteristic feature of GBM is the trend to resist to apoptosis mechanisms. In addition to the activation of mitogenic pathways (Ras-MAPK and PI3K-Akt cascades) and the negative regulation of cell-death signaling (p53 and Rb cascades), other mechanisms to escape cell death are described in GBMs. Expression levels of cell death receptor as CD95, TRAIL, TNFR1 may correlate with the response of GBMs to death-induced apoptosis and with prognosis in consequence. Anti-apoptotic Bcl-2 family proteins (e.g BAK, BAD, BAX, Bcl-XL) showed a role in gliomagenesis: for example Bcl-XL is reported upregulated in association to overexpression of EGFRvIII in GBMs and this situation lead to chemotherapy resistance [5]. Moreover Bcl-2 proteins seem to promote glioma cells migration and invasion causing upregulating MMP2 and MMP3 and downregulating TIMP2 [15].

1.1.5 Target therapies in GBMs

Whenever possible, surgery remains the first approach for GBM patients treatment. In second instance, radio- and/or chemo-therapy (for example using temozolomide, obtaining the best results in *MGMT* methylated cases) are handed out. Taking into account the previous considerations on specific GBM molecular

alterations, many target therapies were tested, but none passed successfully into the clinical practice as main therapy [5, 9]. The difficulty to select an efficient single target agent and high failure rate of target therapies in GBMs are associated to the great variability in GBMs molecular biology. In Table 1.3 and in Figure 1.3 are summarized the current inhibitors used in GBM clinical trials [5, 16].

For example, EGFR inhibitors obtained poor results when handed up as single agent. In fact, the presence of the truncated form EGFRvIII increases tumor susceptibility to EGFR inhibitors only in the case of active *PTEN*, while the loss of *PTEN* is associated to EGFR target therapy resistance [17]. Generally EGFR inhibitors are less effective if PI3K cascade is intact. Other studies indicated that the combinations of EGFR/mTOR inhibitors could have more possibilities even in *PTEN* deficient GBMs [18]. Failure of EGFR target therapy could be also explained by the frequent concomitant presence of alterations in other RTKs (e.g. PDFGR and/or MET) signaling pathways that lead to tumor progression signals, activating Ras/MAPK or PI3K cascades, in spite of EGFR inhibition [5, 10].

The combination of more inhibiting factors represent a possibility to elude the efficacy of a single therapeutic treatment: blocking more targets within the same pathway or in different pathways using multiple agents or a single multi-target agent, could improve the efficacy of the treatment although could enhance the risk of toxic effects [9].

	Inhibitor target	Mono-therapy
RTK	EGFR	Erlotinib, Gefitinib, Cetuximab
	EGFRvIII and amp wtEGFR	mAB 806
	PDGFR	Imatinib, Recentin
	VEGFR and other RTKs	Cediranib (VEGFR, PDGFR, c-Kit) Valatinib (VEGFR, PDGFR, c-Kit) Sunitinib (VEGFR1/2, PDGFR, c-Kit) AEE788 (VEGFR1/2, EGFR) Vandetanib (VEGFR2/3, EGFR) Lapatinib (EGFR, HER2) Sorafinib (RAF, VEGFR2/3, PDGFR, c-Kit) Pazopanib (VEGFR, PDGFR, c-Kit) Tandutinib (FLT3, PDGFR)
Ligand	VEGF	Bevacizumab
Signal Transduction	Akt	Perifosine
	Protein kinase C (PKC)	Tamoxifen Enzastaurin
	mTOR	AP23573 Everolimus Sirolimus Temsirolimus
Protein modification	Histone deacetylase (HDAC)	Suberoylanilide hydroxamic acid
	Farnesyl transferase/RAS	Tipifarnib Lonafarnib
Other	αvβIII integrin	Cilengitide
	Steroid receptors	Synthetic retinoids
	Proteosome	Bortezomib
	Sp1 transcription factor	Tetra-O-methylnordihydroguaiaretic acid
	Topoisomerase I	Irinotecan

Table 1.3. Examples of GBM target therapies. Main targets and corresponding drugs are indicated [5, 16].

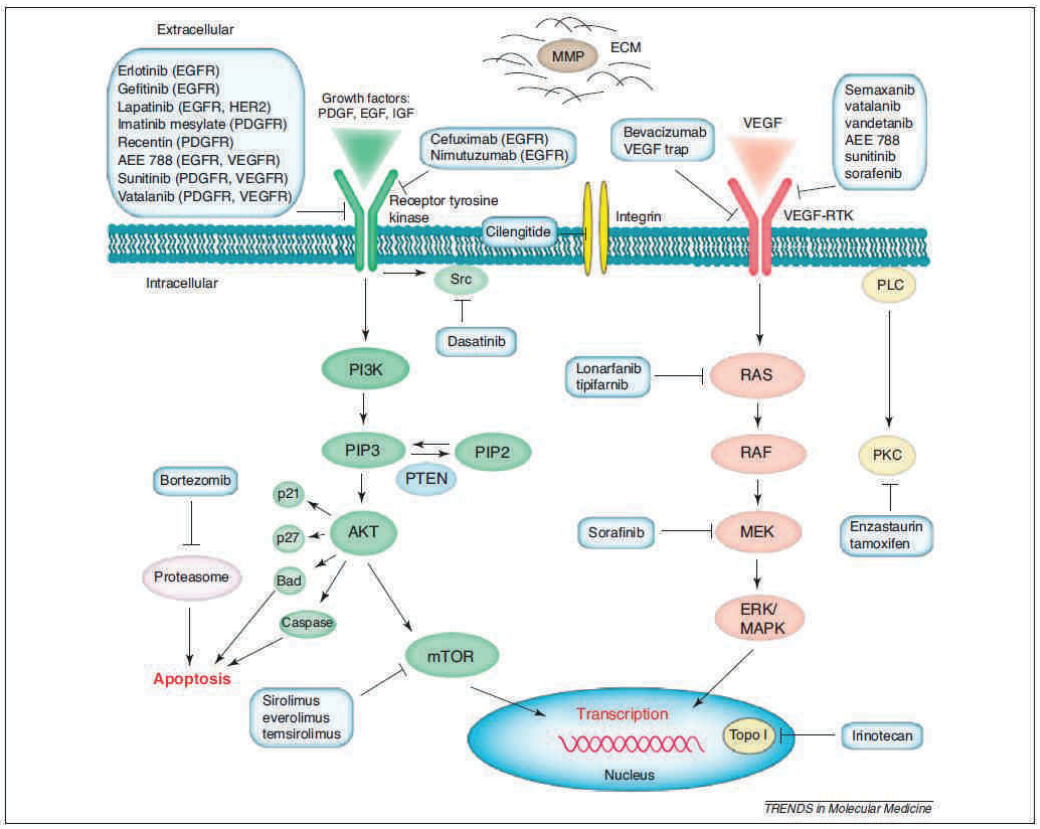


Figure 1.3. Site of actions of main current target therapies for GBM treatment. In the figure are represented the main alterations involved in glioma biology and the relative specific drugs: aberrant oncogenic RTK pathways can be blocked by small molecules and monoclonal antibodies and the consequent activation of PI3K-AKT (green) and RAS (pink) oncogenic pathways are often targeted intracellularly with small molecule inhibitors. Figure obtained from Bai *et al.* [16].

1.1.6 Gene therapy strategies in GBMs

In addition to these target treatments other strategies for GBMs management are developed. For example, gene therapy approaches have demonstrated therapeutic efficacy in glioma xenograft models and had been enrolled in clinical trials [19, 20]. The main strategies are: 1) suicide gene therapy, 2) oncolytic viral therapy, 3) immunomodulatory therapy and 4) nucleic acid therapies using synthetic vectors or nanoparticles [19-21].

The first approach is based on the activation of prodrug *in situ* that blocks DNA synthesis leading to tumor cell division arrest. GBM cells are generally transfected with a viral vector (e.g. herpes simplex virus or adenovirus) which carriers the gene encoding the prodrug activating enzyme. The inactive prodrug, administered systematically, becomes active only where the activating enzyme is

expressed, resulting to specific tumor cell killing. The most studied prodrug is the herpes simplex virus-tymidine kinase (HSV-tk) system which activated the inactive prodrug ganciclovir (GCV) in toxic metabolite [19, 20].

The oncolytic viral strategy takes advantage of replication-competent viral infection which leads to selective tumor cell lysis, with or without gene transfer. Oncolytic herpes simplex virus, conditionally replicating adenovirus and poliovirus are used in antiglioma oncolyitic virus systems [19, 20].

Immunomodulatory gene therapies induce an immune response versus tumor cells: it is obtained through the *in situ* expression of cytokine genes (e.g. IL-2, IL-12, IL-4, INF-γ, INF-β) or through the recruitment of immune cells, such as dendritic cells, to the tumor [19, 20].

The last approach considers the gene therapies based on the use of nanoparticles or synthetic vectors as delivery systems. These vehicles can cross the blood brain barrier (BBB) remaining less immunogenic if compared with viruses: the tumor cells specificity is obtained by conjugation with a driving molecule directed against proteins specifically expressed on tumor cells [22]. DNA plasmids or DNA/RNA oligonucleotides could be delivered across BBB by targeted-nanoparticles. As regards oligonucleotide strategies, antisense oligodeoxynucleotides (AS-ODNs), small interfering RNAs (siRNAs), microRNAs (miRNAs) and nucleic acid aptamers are considered. An example is the antisense strategy versus TGF-β2, a system yet in clinical trial [23]. The base idea of these strategies is to avoid the expression, and therefore preventing protein translation, of target genes involved in tumor progression and survival [19, 20]. While antisense oligodeoxynucleotides (AS-ODNs), siRNAs and aptamers strategies are thought to inhibit specific targets involved in GBMs biology [24, 25], microRNAs strategy are subjugate to peculiar microRNAs expression of the tumor tissue.

MicroRNAs are small endogenous RNAs that are expressed in a tissue specific manner and that are physiologically involved in regulation of several biological processes (see section 1.2). Alterations in microRNAs expression and microRNAs roles in cancer development and progression have been deeply investigated. For these reasons oligonucleotide therapies approaches, based on microRNAs

deliveries, could be focused on silencing or restoration of microRNAs respectively up- or downregulated in GBMs [26-28].

1.2 MicroRNAs: from biogenesis to their functional role in glioblastoma

1.2.1 MicroRNAs biogenesis

MicroRNAs are small non-coding RNAs (19-23 nucleotides) that negatively regulate gene expression by degrading or suppressing the protein translations of mRNAs targets. MicroRNAs sequences represent the 1-3% of whole human genome. They are mainly transcribed by RNA polymerase II, even if some microRNAs could be transcribed by RNA pol III: the long, stem-loop primary transcript (pri-miRNA) is further processed in ~60-70nt pre-miRNA by nuclear Drosha RNase III endonuclease. As represented in Figure 1.4, pre-miRNAs are transported into cytoplasm by Ran-GTP and the export receptor Exportin-5 system: once in the cytoplasm, Dicer RNase III endonuclease, process miRNA precursors into mature 19-23nt miRNA duplexes. These duplexes comprise the mature miRNA and complementary fragment derived from the opposing arm of the pre-miRNA (miRNA*). Only the strand with the less stable hydrogen bonding at its 5'-end is selected (mature miRNA), while the complementary miRNA* is degraded. Mature miRNA is incorporated into ribonucleoprotein (RNP) complexes known as micro-RNPs (miRNPs) or miRNA-induced silencing complexes (miRISCs). The major components of miRNPs are proteins of the Argonaute (AGO) family: these proteins play a central role in binding the mature miRNA and drive it to mRNAs target recognition (Figure 1.4) [29-31].

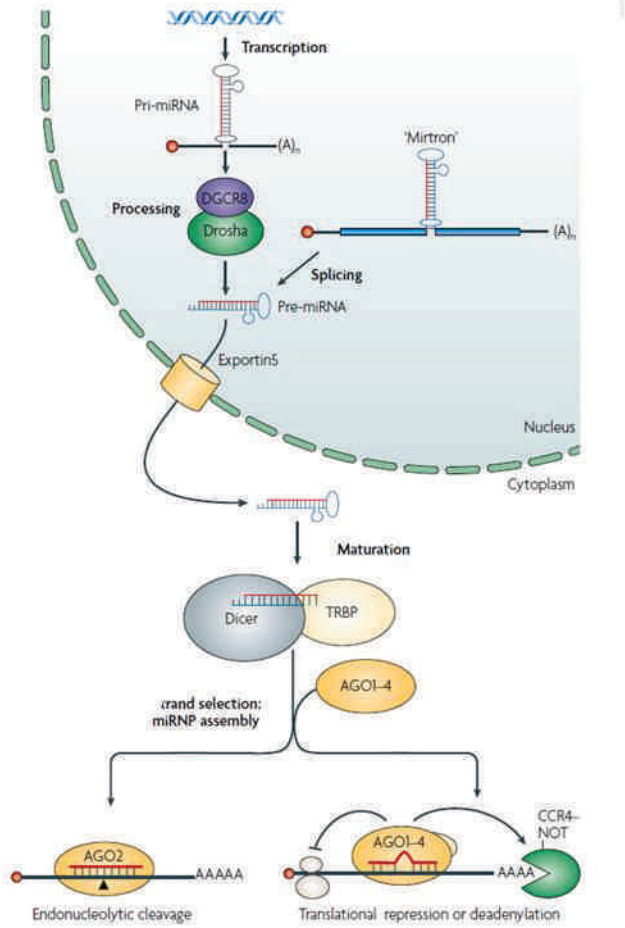


Figure 1.4. MicroRNAs biogenesis. Figure obtained from Filipowicz *et al.* [31].

1.2.2 MicroRNAs mechanism of action

MicroRNA-RISC complexes recognize and link miRNA complementary regions in 3'-UTR of target mRNAs. According to the grade of homology with the mRNAs, microRNAs could act in two ways : a perfect complementarity (mainly in plants) leads to mRNA cleavage, while an imperfect complementarity (mainly in animals) represses mRNA translation with several known mechanisms (Figure 1.5B) [29, 30, 32]. In the latter case, only the miRNA "seed region" is strictly involved in target recognition and binding to mRNA target: a perfect and contiguous base pairing between miRNA nucleotides 2 to 8 and 3'-UTR mRNAs is required (Figure 1.5A, shown in red). In addition, an "A" residue in mRNA sequence corresponding to the position 1 of the miRNA and an "A/U" corresponding to the position 9 (Figure 1.5A, shown in yellow) improve the site efficiency, although they do not need a perfect match with miRNA nucleotides.

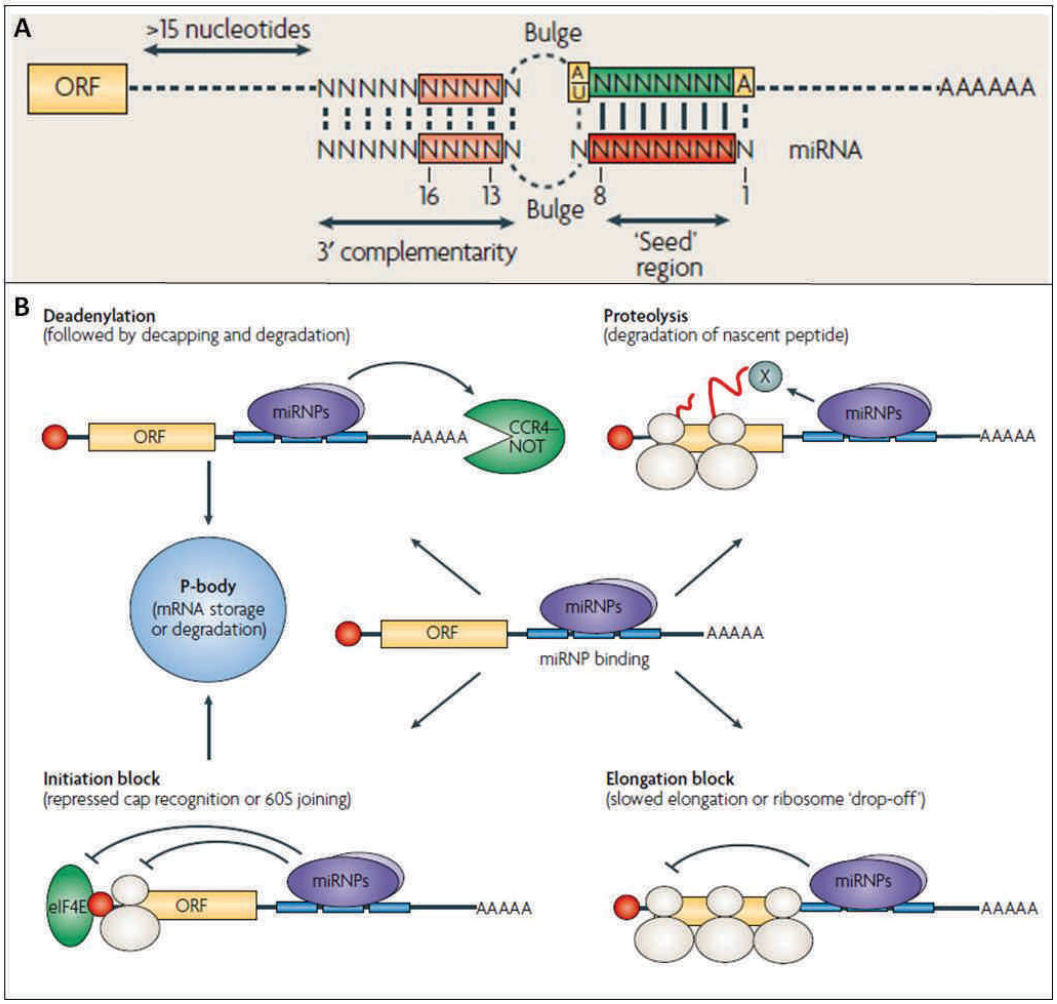


Figure 1.5. MicroRNAs-mRNA binding and post-transcriptional gene repression. A) In animal cells, miRNAs bind their mRNAs targets imperfectly. A perfect complementarity is requested in correspondence of miRNA nucleotides 2 to 8, "seed' region" (shown in dark red and green, on miRNA and mRNA sequences respectively). The presence on mRNA of an A residue corresponding to position 1 of the miRNA, and an A/U corresponding to position 9 (shown in yellow), improve the site efficiency, although they do not need a match with miRNA sequence. A good base pairing to residues 13–16 of the miRNA (shown in light red) is important to stabilize the miRNA-mRNA binding, mainly when matching in the seed region is suboptimal. B) MicroRNAs seem to mediate post-transcriptional repression through 4 main mechanisms. At translation initiation steps miRNPs complexes can induce deadenylation and decay of target mRNAs (upper left), can repress the cap-recognition stage or can block the 60S subunit recruitment (bottom left). mRNAs repressed at the translation-initiation stage are stored/degraded in P-bodies. At post-initiation phases of translation, miRNPs complexes lead to proteolytic cleavage of nascent peptides (upper-right) or to the block of elongation (bottom right). Figure modified from Filipowicz *et al.* [31].

Mismatches must be present in the central region of the miRNA–mRNA duplex, precluding the Argonaute (AGO)-mediated endonucleolytic cleavage of mRNA; finally miRNA 3′ end must have a good complementarity to stabilize the miRNA-mRNA binding, in particular the match with residues 13–16 of the miRNA (Figure 1.5A, shown in orange).

These features lead a single microRNAs to recognize and control thousands of mRNA targets, and thus several cellular processes at the same time, and a single mRNA could be controlled by several microRNAs [33].

Several effects of miRNAs are described on protein expression regulation and in Figure 1.5B the main mechanisms of miRNAs action are summarized. At the initiation step of translation a miRNP complex could induce deadenylation and decay of target mRNAs, prevent the recognition of mRNA 5′-terminal cap by the eIF4E or could repress the recruitment of the ribosomal 60S subunit. The mRNAs not translated because repressed by miRNAs at initiation step, are first stored, and then degraded, in P-bodies. The miRNAs repression on post-initiation steps of translation inducing the degradation of the nascent peptide or the arrest of the elongation through slowing down or 'drop-off' ribosomes [31].

1.2.3 MicroRNAs target prediction

Once clarified the mechanisms of miRNAs action, many studies were focused on understanding their biological function. Their short length and their ability to bind several mRNAs with imperfect complementarity, have led to the necessity of the develop of useful predicting bioinformatics tools to find their regulatory targets. Available algorithms are based on: 1) imperfect complementarity between miRNA seed region and 3'-UTR of mRNA target; 2) evolutionary conservation of the miRNA recognition elements; 3) thermo-dynamic stability of the miRNA-mRNA heteroduplex; 4) mRNA sequence features outside the target site [34].

The main available prediction tools for mammalians and their principle characteristics are listed and summarized in Table 1.4. It is reported that prediction algorithms based on stringent Watson–Crick seed pairing have the highest specificity, sensitivity and superior predictive power (e.g. TargetScan, and PicTar which have also a high degree of overlap) [34, 35]. It is difficult to define

if a tool could be superior to another one and it is a common practice using different tools at the same time, considering the intersection of their results [36-39].

Moreover, depending on the scope of each research study, the analysis of the only experimentally validated targets relative to selected miRNAs could be useful. For this goal, other web prediction tools are available: for example miRecords [40], miRTarBase [41] and miRWalk [42].

MiRecords and miRTarBase include manually curated experimental interactions based on literature surveying and the experimental techniques used for the validation of each microRNA target are ever shown, while miRWalk is based on an automated and extensive text-mining search performed on PubMed database [40-43]. Therefore this last database includes more interactions than the other two, but often less reliable [40-43] and, even in this case, it is common practice consider the results intersection of more than one tool at the same time.

1.2.4 MicroRNAs and cancer

Due to their key role in the regulation of gene expression, in the last years miRNAs tissue specific presence, quantification and functional analysis had been deeply investigated to understand their peculiar involvement in cellular processes. It is now established that each tissue shows a characteristic microRNAs expression pattern which could be modified in association with a number of different diseases including neoplastic transformation [28, 44, 45]. It is now found that microRNAs could represent important cancer biomarkers, both because their expression patterns are tissue specific and mainly because they play a role in important cellular processes involved in the tumorigenesis and progression of several cancers, including proliferation, invasion, migration and angiogenesis [28, 44-46]. Many studies have been conducted to define specific microRNAs signatures of tumors in several organs, as for example lung [47], pancreas [48], prostate [49], thyroid [50], breast [51], melanoma [52], colon [53] and blood [54]. The common aim of these studies was to characterize each cancer for miRNAs expression in correlation with tumor initiation, progression to higher stages of malignancy or with patient survival.

Target prediction algorithm	Features		Assessment		Website
	Criteria for prediction and ranking	Cross-species conservation	Advantages	Disadvantages	
miRanda	Moderately stringent seed pairing, site number, pairing to most of the miRNA, free energy binding	conservation filter is used	beneficial for prediction sites with imperfect binding within seed region	low precision, too many false positives	microrna.org
TargetScan	Stringent seed pairing, site number, site type, site context (which includes factors that influence site accessibility)	option of ranking by likelihood of preferential conservation rather than site context	- many parameters included in target scoring - final score correlates with protein downregulation	sites with poor seed pairing are omitted	targetscan.org
PicTar	Stringent seed pairing for at least one of the sites for the miRNA, site number, overall predicted pairing stability, binding energy	required pairing at conserved positions	miRNAs with multiple alignments are favored	does not predict non-conservative sites	pictar.mdc-berlin.de
DIANA-microT	Moderately stringent seed pairing, site number, free energy binding	dataset of conserved UTRs among human and mouse is used	possibility of using own miRNA sequence as an input	some miRNAs with multiple target sites may be omitted	diana.cslab.ece.ntua.gr/microT
PITA	Moderately stringent seed pairing, site number, overall predicted pairing stability, predicted site accessibility, binding energy	user-defined cut-off level	the secondary structure of 3'UTR is considered for miRNA interaction	-ow efficiency compared to other algorithms	genie.weizmann.ac.il/pubs/mir07/mir07_data.html
Rna22	Moderately stringent seed pairing, matches to sequence patterns generated from miRNA set, overall predicted pairing and predicted pairing stability, free energy	not included	allows to identify sites targeted by yet undiscovered miRNAs	low efficiency compared to other algorithms	cbcsrv.watson.ibm.com/rna22.html

Table 1.4.: Main MicroRNAs prediction tools. Table revised by Bartel et al. [35] and Witkos et al. [39].

In tumors some microRNAs seem to act as oncogenes, also called "oncomir": they are generally highly expressed in tumor tissues and they target genes with tumor suppressor activities leading to their significant downregulation, or indeed they regulate other genes involved in cell differentiation. In this manner an overexpression of oncogenic miRNAs could be related to uncontrolled proliferation, resistance to apoptotic signals, neovascular formation and cell invasion and migration [44]. An example of a miRNA that acts as oncomir in several cancers is miR-21: its main targets are *PTEN*, *PDCD4* and p53 and TGFβ network components [55].

On the other hand, several other miRNAs have been found to be weakly expressed or absent in tumors compared to normal tissue and they act as tumor suppressors. Their main targets are oncogenes and/or genes that control cell differentiation or apoptosis, that result overexpressed, with obvious advantages for cancer progression [44]. An example of a tumor suppressor miRNA could be miR-34a which mainly regulates genes like *MYCN, BCL2, SIRT1, CAMTA1, NOTCH1, JAG1, CCND1, CDK6* and *E2F3* [56].

1.2.5 MicroRNAs and glioblastoma

In the last years several studies have been performed in attempt to identify a specific microRNAs expression pattern of GBM [57-76] and a small subset of consistently deregulated miRNAs were further functional characterized for their activities and downstream targets possibly involved in this tumor [73, 77-81].

Recent works of LeBrun *et al.* [27] and Mizoguchi *et al.* [82], for example, had tried to summarize the results of many GBM miRNAs profiling studies: underlying the techniques adopted, the source of tumor and control samples analyzed in each study, they focused their attention on miRNAs found consistently deregulated. The main microRNAs profiling studies published on GBM are summarized in Table 1.5. It is evident the great variability among the different studies, going from the technique used for the number of samples analyzed, through the non-neoplastic control enrolled and the number of target microRNAs studied and finally to miRNAs expression results [27, 82].

Profiling study	Method	miRNAs analyzed	Samples	Upregulated miRNAs	Downregulated miRNAs
Chan *et al.* 2005 [57]	Microarray	180	3 fresh GBMs, versus 8 NBs, 6 GBM cell lines versus NBs	miRNA-**21**, 138, 347, 135, 291-5'	miRNA-198, 188, 202
Ciafrè *et al.* 2005 [58]	Microarray	245	9 fresh GBMs versus 9 ANBs	miRNA-**10b**, 130a, **221**, 125b-1, 125b-2, 9-2, **21**, 25, 123	miRNA-**128a**, 181c, 181a, **181b**
Rao *et al.* 2010 [63]	LNA Microarray	756	26 fresh GBMs, 13 AAs, 7 NBs	miRNA-**21**, 146b-5p, **155**, **16**, **193a-3p**, 199a/b-3p, 335, 142-5p, 34a, 513a-5p, **451**	miRNA-**126,** 22, 143, 381, **24,** 552, 886-5p, **128**, 509-3-5p, 376c, 886-3p, 219-2-3p
Godlewski *et al.* 2008 [62]	Microarray	245	Unspecified # fresh GBMs versus ANBs	miRNA-383, 519d, **21**, 516-35p, 26a, **10b**, 486, **451**	miRNA-124a, 137, **323**, 139, **218, 128-2**, 483, **128-1**, 299, 511-1, 190
Zhou *et al.* 2010 [65]	Microarray	435	5 GBM cell lines, 1 AA cell line, and 1 NB	miRNA-137, 23b, 23a, 222, **221**, 106, **15b**, **21**	miRNA-451, **124, 495,** 223, **329, 126,** 219, 1, **330,** 342, **323**, 127, **128**, **132, 95**
Conti *et al.* 2009 [60]	RT-PCR assay	8	10 fresh GBMs, 10 AAs, 8 LGAs, versus 4 NBs	miRNA-**21**, **221**	miRNA-**181b**
Silber *et al.* 2008 [59]	RT-PCR assay	192	Unspecified # of fresh GBMs, AAs versus NBs	miRNA-**21**, **155, 210**	miRNA-101, **128a**, **132,** 133a, 133b, 149, **153,** 154*, 185, 29b, **323**, 328, **330**
Gal *et al.* 2008 [61]	Microarray	NS	CD133+ (stem) vs. CD133- (non-stem) cells from 6 GBM tissues	miRNA-**451**, 486, 425, **16**, 107, 185	None found
Guan *et al.* 2010 [67]	TaqMan array	365	8 fresh GBMs versus 4 AAs	miRNA-**15b**, **21**, 135(b), 196a, **196b**, 363	miRNA-105, **128-2**, 184, 302b, 302d, 367, 383, **504,** 517c, 601
Sasayama *et al.* 2009 [73]	Microarray	127 or 188	3 fresh GBMs versus ANB	miRNA-**10b**, **21**, **92b**, 106b, 183	miRNA-134, 302c, **329,** 369-3p, **379**
Malzkorn *et al.* 2009 [71]	RT-PCR assay	157	4 fresh secondary GBMs versus DAs	miRNA-9, 15a, **16**, 17, 19a, **20a**, **21**, **25**, 28, 130b, 140, **210**	miRNA-184, 328
Zhang *et al.* 2013 [76]	Microarray	1146	82 fresh GBMs vs 5 NBs	miRNA-518b, 566	miRNA-181d, 524-5p, 1227
Lang *et al.* 2012 [69]	Microarray and NGS	NS	3 GBM stem cell lines vs 3 normal neural stem cell lines	miRNA-10a, **10b**, 140-5p, 204, 424, 34a, **193a-5p**, 455-5p	miRNA-**124,** 874
Hua *et al.* 2012 [68]	NGS	875	3 fresh GBMs vs 3 NBs	Top 10 upregulated: miRNA-**10b**, 96, 10b*, 182, 135a*, 21*, **21**, 542-3p, **148a**, **92b**	Top 10 down-regulated: miRNA-, 433, 7-1*, 129*, 628-5p, 935, **218,** 31, 876-3p, 1258, **132**
Slaby *et al.* 2010 [64]	RT-PCR assay	8	22 FFPE-dissected GBMs vs 6 NBs	miRNA-**21**	miRNA-**181b**, 181c, **221**, **222**, **128a**

Table 1.5 - continued

Profiling study	Method	miRNAs analyzed	Samples	Upregulated miRNAs	Downregulated miRNAs
Loftus *et al.* 2012 [70]	Microarray	NS	7 GBM cell lines: migrating cells vs non migrating cells	miRNA-99a*, 767-3p, 202*, 556-3p, 655, **451,** 495, 579, 223, 381, 329, 769-3p,524-3p, 93*, 220a, 491-5p,200c*, 133b, 19b-1*, 520h, **92b**, 657, 891a, 326, 541*	miRNA-16, 30c, 15b, 23b, 103, **107, 24,** 93, 15a, 151-5p, 23a, 30b, 30a, 29a, 455-3p, 25, 106b, 92a, 125b, 125a-5p, **222**, 17, 20a, 29c, 26a, 574-3p, **181b,** let-7a, let-7f, let-7e, let-7b, let-7c, let-7d, let- 7g, let-7i
Srinivasan *et al.* 2011 [75]	Microarray	305	222 GBMs vs 10 NBs (data from TGCA)	miRNA-**20a**, 106a. 17-5p, **148a,** 146b, 200b, **193a**	miRNA-**221, 222**, 31
Skalsky *et al.* 2011 [74]	NGS	NS	6 fresh GBMs vs 3 NBs	miRNA-10b*, **10b**, 891a, 93, **196b**, 21*, 320d, 217, 4448, **25**, 660, **21**, 320c, 296-3p, **92b**, 10a, 92a, let-7i*, **148a***	miRNA-**124, 95, 132,** 139-5p, 7, 543, let-7d, 323-3p, **128,** 598, 103a, 103b, 139-3p, 487b, 873, 323b-3p, 138-1*, 301b, **107,** 411-3p, 124*, 342-3p, 379*, 212, let-7g, **153,** 181d, 22, 889, 885-5p, **379,** 138, let-7e, **218, 221,** 136, **24,** 4787-3p, **126,** 548i, 382, 1270, **495,** 2392, 1273d, 767-5p, 770-5p, **504,** 490-3p
Niyazi et al . 2011 [72]	Microarray	>1000	35 FFPE GBMs: short term vs long term survivors	miRNA-3163	miRNA-539, 1305, 1260, let-7a
Dong *et al.* 2010 [66]	Microarray	534	240 GBMs vs 10 NBs f(data from TGCA)	miRNA-141, **21, 155**, 27a, 93, 23a, **196b**, 629, 96, 106b, 492, 200b, 205, 449, **25**, 130b, 135b, 422a, 196a, **15b**, 17-3p, 92, **210,** 296, 320, 362, 200c, 200a, 17-5p, 500, 106a, 30a-5p, 15a, let-7i, 130a, 34c, 450, 532, **92b**, 224, 200a*, 199b, **20a**, 503, **193a,** 605, 373*, 452*, **16**, 24, 34b, 505, 502, 602, 215, 142-3p, 660, 590, 610, 367, 18a, 542-3p, 527, 20b, 195, 339, 214, 142-5p, 19b, 422b	miRNA-129, **218,** 124a, **329, 323,** 7, 628, 139, 137, 491, **379,** 138, 410, 485-5p, **128a,** 128b, 154*, **495,** 769-5p, 582, **132,** 433, 103, 598, 326, **107,** 29b, 338, **153,** 330, 539, 411, 381, 432, 1, 154, 656, 383, **95,** 299-5p, 127, 758, 29c, 203, 490, 342, 136, 377, 517c, 376a*, 380-3p, 409-5p, 219, 496, 448, 369-5p, 149, 340, 133b, **504,** 324-5p, 487b, 577, 485-3p, 133a, 488, 592, 409-3p, 346, 331, 33, 487a, 382, 369-3p, 125a, 29a, **221**

Table 1.5 (previous pages). MicroRNAs profiling studies in glioblastoma. GBM: glioblastoma, AA: anaplastic astrocytoma, NB: normal brain, ANB: adjacent normal brain, DA: diffuse astrocytoma, NS: not specified, NGS: next generation sequencing, TGCA: The Cancer Genome Atlas (TCGA) Datasets, FFPE: Formalin-Fixed, Paraffin-Embedded, Bold: common dysregulated miRNAs (in at least 3 reports).

From this comparison, only few miRNAs (upregulated: miR-21, 10b, 221, 16, 451, 193a, 148a, 92b, 196b, 25, 155, 15b and 210; downregulated: miR-128, 128a, 323, 124, 218, 132, 107, 221, 222, 181b, 95, 153, 379, 126, 495, 504, 329 and 330, in bold in Table 1.5) shared the same deregulation pattern in at least 3 studies reported. Interesting to note that miR-221/222, defined as oncomirs in GBM and deeply discussed by several works as important microRNAs in GBM pathogenesis [60, 83-87], were found both up- and downregulated in at least 3 profiling studies (Table 1.5).
Taking in consideration this summary of some of the most cited miRNAs profiling studies (Table 1.5), it is clear that by now an exhaustive microRNAs profile of glioblastoma is far to be well defined.

Many other works were focused on a single, or a small subset of microRNAs to study their function, genetic regulation and expression in GBM tissues/cell lines. These functional studies were been very important to validate possible targets of those microRNAs found deregulated in many previous profiling works, clarifying their molecular roles in GBM pathogenesis with the aim to identify putative prognostic or therapeutic biomarkers [26, 46, 56, 57, 59, 61, 62, 65, 67, 73, 77-81, 87-100]. For example Karsy *et al.* [88] and Møller *et al.* [101] have summarized the main miRNAs studied in GBM, reporting their functional targets and their putative role in tumorigenesis (Table 1.6).
Finally, taking into account the previous considerations on molecular alterations involved in glioblastoma pathogenesis (see paragraph 1.1.3), Figure 1.6 represents few examples of how miRNAs could regulate glioblastoma cell proliferation and survival, targeting molecules belonging to the principal oncogenic pathways discussed above.

miR	Functions	Targets shown in GBM[a]	Expression pattern in GBM
miR-7	Differentiation, invasion, proliferation	EGFR, IRS1, IRS2	Down
miR-9/miR-9*	Proliferation, self-renewal	SOX2	
miR-10a/miR-10a*/ miR-10b	Apoptosis, autophagy, chemoresistance, invasion, prognosis, proliferation, senescence, tumor growth	BCL2L11/Bim, TFAP2C/ AP-2g, CDKN1A/ p21, CDKN2A/p16, HOXD10	Up
miR-15b	Cell cycle, proliferation	CCNE1	Heterogeneous
miR-17-92	Cell cycle, proliferation	CDKN1A/p21, E2F1, PTEN, CTGF	Up, down in $CD4^+$ T-c from GBM patients
miR-21	Apoptosis, chemoresistance, invasion, proliferation, tumor growth	PTEN, RECK, PDCD4, TIMP3, BCL2, SPRY2, TIMP3, PDCD4, MTAP, SOX5, JMY, TGFRBR2, TGFBR3, TP73L, APAF1, BMPR2, TOPORS, DAXX, TP53BP2, PPIF	Up
miR-26a/miR-26b	Apoptosis, proliferation, tumor growth, vasculogenic mimicry	PTEN, MAP3K2, IFNB, EphA2, Rb1	Up (genetic amplification)
miR-29b	Apoptosis, invasion, proliferation	PDPN	Low
miR-34a	Apoptosis, cell cycle, differentiation, invasion, proliferation, tumor growth	MET, NOTCH1, NOTCH2, CDK6, MYC, SIRT1	Down (reduced by los of p53)
miR-93	Neoangiogenesis, tumor growth	Integrin-β8	Unknown
miR-101	Invasion, neoangiogenesis, proliferation, tumor growth	EZH2	Down
miR-124a	Cell cycle, differentiation, invasion, proliferation	CDK6, SCP1, PTBP1, ITGB1, LAMC1, IQGAP1, pRB	Low
miR-125a/miR-125b	Apoptosis, invasion, proliferation	PDPN, BMF	Low (miR-125a)
miR-128	Proliferation, self-renewal, tumor growth	EGFR, BMI1, E2F3A, ARP5	Low
miR-137	Cell cycle, differentiation, proliferation	CDK6, MITF, EZH2	Low (methylated)
miR-146b-5p	Invasion, migration	EGFR	Low
miR-153	Apoptosis, proliferation	Bcl-2, Mcl-1	Low
miR-181a/miR-181b/miR-181c	Apoptosis, colony formation, invasion, proliferation, radiosensitivity	Bcl-2	Low
miR-196a/miR-196b	Survival		High
miR-218	Migration	IKK-β	Low
miR-221/miR-222	Apoptosis, cell cycle, CTL-mediated tumor lysis, invasion, proliferation	NIAP, p27/KIP1, p57/ KIP2, STAT1, STAT2, PUMA, ICAM1	High
miR-296	Neoangiogenesis	HGS	High (endothelial cells)

Table 1.6 - continued

miR	Functions	Targets shown in GBM[a]	Expression pattern in GBM
miR-302-367 cluster	Differentiation, invasion, proliferation, self-renewal, stemness	CXCR4/SDF1	High (serum-mediated differentiation)
miR-326	Apoptosis, invasion, metabolism, proliferation, tumor growth	NOTCH1, NOTCH2, PKM2	Low (reduced by notch signaling)
miR-339	CTL-mediated tumor lysis	ICAM1	High
miR-381	Proliferation	LRRC4	High
miR-451	Apoptosis, cell cycle, chemoresistance, invasion, proliferation, proliferation/migration regulation	CAB39, MIF, UBE2H, ARPP19, GATA2, ABCC1, AKT1, CCND1, MMP-2, MMP-9, Bcl-2, SMAD3, SMAD4	High (glucose regulated)
let-7a	Migration, proliferation	Pan-RAS, N-RAS, K-RAS	Low

Table 1.6. Dysrewgulated miRNAs and their role in GBM [88].

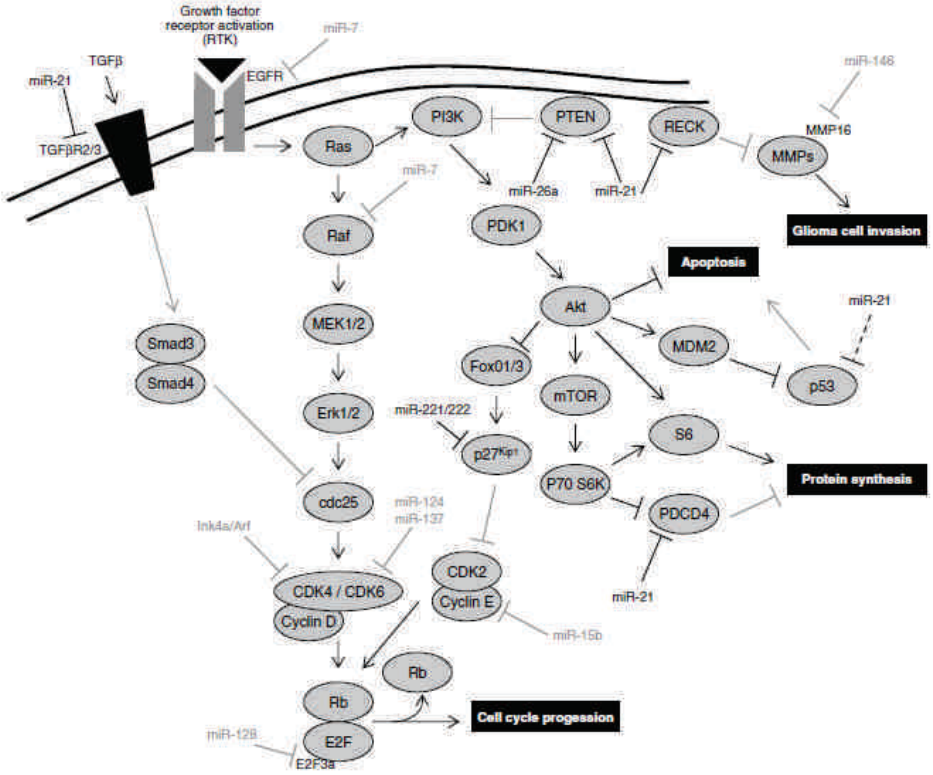


Figure 1.6. Examples of microRNAs actions on oncogenic pathways involved in GBM. Upregulated miRNAs are indicated black, downregulated in gray. The dotted arrow indicates an indirect control of miR-21 on p53 pathway [102].

1.2.6 Experimental strategies for miRNA expression analysis

A typical microRNAs expression study starts from the choice of the type of sample to analyze, the small or broad subset of miRNAs to investigate and the most adequate method to apply. The overview of microRNAs expression studies in GBMs is extremely heterogeneous, from the experimental set up to results obtained (Table 1.5).

As regards starting material, the common choice spread between Fresh/Frozen tumor tissues [57-60, 62, 63, 67, 68, 71, 73, 76] and glioblastoma cell lines [61, 65, 69, 70, 77, 79, 81]. As reported in Table 1.5 only two studies have adopted formalin fixed and paraffin embedded (FFPE) samples as starting material [64, 72]. The main pros of fresh tissue or cell lines choices is the large quantity and the best quality of extracted RNA. On the other hand, the main cons of using cell lines to investigate microRNAs expression is that they could not reflect the real biological and physiological conditions of GBM: both because they are immortalized and they have undergone to molecular modifications which are not specific of the tumor in analysis and moreover for the lack of microenvironment influence on several steps of tumor progression. Therefore, the use of cell lines for profiling studies could result in not reliable data, mainly if compared to the same obtained on tumor tissues, but they remain an important resource for miRNAs functional studies in vitro. The main cons of using Fresh/Frozen specimens is the difficulty to estimate the total amount of neoplastic cells in the starting tissue. In fact, even if a first evaluation of neoplastic cells is usually performed on a snap frozen section, miRNA/RNA fractions are extracted from an unchecked piece of tumor tissue. In this latter it is not possible to determine if the percentage of tumor cells is maintained or how much is the normal cells contamination, due to lymphocytes, endothelial or normal glial cells, that could surely influence expression data.

It has been reported that microRNAs are not influenced by degradation due to formalin fixation as longer RNAs both for their short length (19-25nt) and because they are protected by Argonaute protein complex [103-105]. Previous studies, focused on different tissues as liver [106], colon [106], prostate [107, 108], lymphatic tissue node [109, 110] or breast [111], had shown the possibility

to analyze microRNAs expression starting from FFPE samples. Many of them have compared microRNAs expression in paired Fresh/Frozen and FFPE specimens obtaining good correlation values indicating that FFPE tissues are suitable for miRNAs analysis and that the obtained results are similar to those obtained starting from Fresh/Frozen samples [106, 108, 109]. Once established that the putative main cons of starting from FFPE tissues, that was a possible limitation in miRNA/RNA extraction, seems to be overcome, several pros are to take in consideration: a large number of cases could be retrieved form anatomic pathology archives, neoplastic areas could be dissected ensuring a good enrichment in neoplastic cells and the possibility to retrieve normal brain tissue is higher than fresh samples.

The number of miRNAs to analyze is an arbitrary selection and influences the choice of the most appropriate method to use. The most common techniques used in miRNAs expression profiling studies are: microarray expression analysis, real-time qPCR and next generation sequencing (Table 1.5).

Microarray analysis, as next generation sequencing techniques (Genome Analyzer - Illumina Inc or Genome Sequencer FLX - 454 Life Science and Roche Applied Science), let the investigation of a large number of different microRNAs at the same time leading to a wide panorama of microRNAs expression in a selected tissue. Furthermore, deep sequencing strategies, analyzing thousands sequences in parallel from a given sample, could highlight both miRNAs present in few copies and could discover novel miRNAs without the limitation of microarray probe selection. These high throughput assays are useful to have a broad overview of the presence and regulation of microRNAs. However the subset of interesting miRNAs resulted from these preliminary analysis, needs to be validated by miRNA-specific approaches like Northern Blotting, in situ hybridization (ISH) or real-time qPCR [112].

Northern blotting is useful to validate specific miRNAs, but it is laboratory-intensive, time consuming, poor sensitive and requires a large amounts of starting material [112].

In situ hybridization (ISH) is the unique technique which permits simultaneously to visualize and localize the presence of a specific miRNA in tissue specimen cells, but it is not a quantitative assay [112].

Quantitative real-time PCR (qRT-PCR) is considered the gold standard for quantification of nucleic acid levels and have been widely applied to miRNA investigations: it remains the most used techniques for validating microarray/NGS results and furthermore it is also used to study miRNAs expression profiles analyzing a selected pattern of miRNAs (Table 1.5). It is efficiency, relatively low cost, it could start also from low quantity of starting material, and both high and low abundance miRNAs can be detected [112].

Chapter 2:

Thesis purposes

2.1 PERNO project

The PERNO project (Progetto Emiliano-Romagnolo di Neuro-Oncologia, www.perno.it) was launched in 2008 and finished at the end of 2012. It involved four universities of Emilia Romagna region (University of Bologna, Ferrara, Modena Reggio-Emilia and Parma) and all region local health services (Bologna, Ferrara, Forlì-Cesena, Modena-Carpi, Parma-Fidenza, Piacenza, Ravenna, Reggio-Emilia and Rimini) gave their precious contribute to the project.

The main purpose of the this project was to collect primary brain tumor samples and their relative epidemiologic data, about prevalence, incidence and prognosis. Specific research studies were developed within the PERNO project, all of them in strict connection with each other and with professional partnership of neurologists, neuroradiologists, neurosurgeons, oncologists, radiotherapists, pathologists and molecular biologists.

The main sub-projects were :

1- *Diagnostic and therapeutic courses of glioblastoma patients*: its main aim was to evaluate health care course and follow up of glioblastoma patients to guarantee them a better assistance;

2.- *Diagnostic and prognostic value of Neuro-Imaging in primitive brain tumors*: its main aim was to set up non-invasive imaging techniques (e.g. TAC) to promptly identify the tumor type and stage, and to promptly recognize possible recurrences.

3- *Molecular alterations potentially useful for glioblastoma diagnosis and treatment*: it consisted of several research studies all involved in the identification of molecular alterations in glioblastoma patients, useful as potentially biomarkers for target therapies or to improve diagnosis and prognosis definition.

4- *Epilepsy in primitive brain tumors*: its main aim was to recognize which factors are directly related to seizures appearance in glioblastoma patients, and to clarify the prognosis for these patients looking for the most adequate therapies.

In detail the third part of the PERNO project was further organized in 5 sub-categories:

3a- Expression analysis of IGFBP2 and valuation of its levels in patient sera;

3b- Analysis of mtDNA molecular alterations and their possible involvement in tumor development, progression, chemo- and radio- resistance;

3c- Expression analysis of a subset of microRNAs which could have a functional importance in high grade gliomas;

3d- Polymorphisms analysis of DNA repair genes and their possible involvement in chemo- and radio-resistance;

3e- Role of ubiquitine-proteasome system in the development of primitive brain tumors.

Among them my PhD research program was based on sub-project "3c". At the beginning of the study, we selected 19 microRNAs known to play a role in glioblastoma, according to data yet published in literature (Table 2.1).

The main aim was to perform the expression analysis of this pattern of microRNAs in brain tumors collected within the PERNO project, in order to define and to evaluate a possible "microRNAs signature" of glioblastoma. This microRNAs profile could be helpful to identify biomarkers potentially useful for prognosis, diagnosis or target therapy strategies.

This present study was performed in the molecular laboratory of Anatomic Pathology division of the Bellaria Hospital (University of Bologna). This structure represented the case collection center for the PERNO Group: all cases retrieved from collaborators were collected in our lab, nucleic acid extractions and quantifications for DNA, RNA and microRNAs were performed and DNA/RNA aliquots or sample tissues were distributed to PERNO partners.

miRNA	Chromosomal localization	Expression level in GBM [Ref.]	
miR-7	9q21.3	DOWN	[59, 74, 79, 100]
miR-9	1q22	UP	[58, 71, 96]
miR-9*	1q22	UP	[58, 71, 96]
miR-10a	17q21.32	UP	[99]
miR-10b	2q31.1	UP	[58, 59, 62, 91]
miR-17	13q31.3	UP	[71]
miR-20a	13q31.3	UP	[71]
miR-21	17q21.31	UP	[58, 59, 62, 71]
miR 26a	3p22.2	UP	[78]
miR-27a	19p13.13	UP	[78, 90]
miR 31	9p21.3	DOWN	[59]
miR-34a	1p36.22	DOWN	[80, 94]
miR-101	1p31.3	DOWN	[59, 81]
miR-137	1p21.3	DOWN	[59, 62]
miR-182	7q32.2	UP	[78, 113]
miR-221	Xp11.3	UP	[58, 60, 95, 114]
miR-222	Xp11.3	UP	[114]
miR-330	19q13.32	DOWN	[59]
miR-519d	19q13.42	UP	[62]

Table 2.1. **MicroRNAs chosen for this project**. Their chromosomal localization and their regulation in GBM is indicated

Chapter 3:

Materials and Methods

3.1 Ethic Statement

The study was approved by Ethic Committee of Azienda Sanitaria Locale di Bologna (number of study 08075, protocol number 139/CE of 5th February 2009, Bologna, Italy). All patients signed a written consent for molecular analysis and for anonymous data publication for scientific studies and all information regarding the human material used in this study was managed using anonymous numerical codes.

3.2 Selection of cases

3.2.1 Glioblastoma group

Fifty cases of glioblastoma were retrieved at Bellaria (institute of Anatomic Pathology, Bologna, Italy) and Bufalini (institute of Anatomic Pathology, Cesena, Italy) Hospitals, within PERNO (Progetto Emiliano-Romagnolo di Neuro-Oncologia) project. All specimens were primary GBMs and patients had not undergone neoadjuvant therapy before surgery. Patients were 27 males and 23 females, aged 41 to 78 years (mean 61.9 yrs). All 50 samples were diagnosed as GBM according the 2007 WHO criteria [4].

The specimens were collected within one hour after surgery and immediately a snap-frozen section was performed. A pathologist evaluated snap-frozen sections for each case in order to verify if the tissue was representative of a ‘‘high-grade glioma’’. Each sample was divided into three specimens: 1) one fresh specimen was incubated in RNA later solution (Applied Biosystem, Austin, TX, U.S.A.) for 1 hour at room temperature and stored at -80°C after quick-frozen in liquid nitrogen (Fresh/Frozen tissues); 2) one fresh specimen followed an alternative procedure through under-vacuum treatment in plastic bags; 3) a third fresh specimen was directly stored at -80°C after quick-frozen in liquid nitrogen. The remaining tissues were routinely processed, formalin fixed and paraffin embedded (FFPE), for routine histological analysis.

3.2.2 Normal adjacent the tumor group

Normal adjacent the tumor tissues were retrieved at a distance between 1 and 2 cm from the margin of 15 primary FFPE GBMs. Patients were 8 males and 7 females, aged 50 to 75 years (mean 62.7 yrs), all belonging from GBM group previously described.

3.2.3 Epileptic group

Fifteen polar temporal cortical FFPE specimens, removed in patients submitted to surgery (tailored polar anterior temporal resection along with uncus-amygdalohippocampectomy) for drug-resistant epilepsy, were randomly selected from the archives of the Anatomic Pathology of Bellaria Hospital. Epileptic patients were 7 males and 8 females, aged 25 to 52 years (mean 39.7 yrs). All of them presented drug-resistant anteromedial temporal lobe epilepsy. Histologically, eleven cases showed focal cortical dysplasia while four patients had hippocampal sclerosis. None of them were affected by a neoplastic lesion, including GBM. The tissue used for microRNAs extraction was taken from the temporal lobe cortex.

3.2.4 Grade III Group

Fifteen cases of grade III gliomas were collected (2 anaplastic ependymomas, 7 anaplastic oligodendrogliomas, 3 anaplastic astrocytomas, 3 anaplastic oligoastrocytomas). Patients were 10 males and 5 females, aged 30 to 74 years, mean 50.1 yrs. All samples were diagnosed according the 2007 WHO criteria [4].

3.2.5 Grade II Group

Fifteen cases of grade II gliomas were collected (2 ependymomas, 7 oligodendrogliomas, 1 pleomorphic xanthoastrocytoma, 1 astrocytoma and 4 neurocytomas). Patients were 11 males and 4 females, aged 21 to 74 years, mean 42.8 yrs. All samples were diagnosed according the 2007 WHO criteria [4].

3.2.6 Grade I Group

Fifteen cases of grade I gliomas were collected (4 pilocytic astrocytomas and 11 gangliogliomas). Patients were 6 males and 9 females, aged 2 to 35 years, mean 20.8 yrs. All samples were diagnosed according the 2007 WHO criteria [4].
The grade I, II and III brain tumors were all randomly selected from the archives of the Anatomic Pathology of Bellaria Hospital.

3.2.7 Commercial reference

The FirstChoice® Human Brain Reference RNA was purchased from Ambion (Ambion, Austin, TX, U.S.A.). According to the manufacturers' data sheet, this was a pool of RNAs obtained from several normal brain regions of 23 healthy donors, 13 males and 10 females, aged 23 to 86 (mean 69.7 yrs). FirstChoice® is certified to contain small RNAs, including microRNAs.

3.2.8 Cell lines

Cell lines of prostate carcinoma (LNCaP, CRL-1740), breast adenocarcinoma (MCF7, HTB-22) and glioblastoma (U-87 MG, HTB14), provided by American Type Culture Collection (ATCC, Rockville, MD, USA), were used for evaluating efficiency of primers per each miRNA analyzed.

3.3 Nucleic acid extractions

3.3.1 DNA extraction

Fresh/Frozen samples were processed for DNA extraction using MasterPure™ DNA Purification Kit (Epicentre, Madison, WI, U.S.A.), according to manufacturer's protocol. DNA was extracted starting from 20 to 50 mg of tissue and quantified using the Qubit fluorometer (Invitrogen, Carlsbad, CA, U.S.A.).

3.3.2 RNA and microRNAs extractions

Fresh/Frozen specimens and cell lines were processed for microRNAs extraction using mirVana miRNA isolation kit (Applied Biosystem, Austin, TX, U.S.A.). Briefly, total RNA was extracted and small RNA fraction enriched starting from

50 to 80 mg of tissue or 3 millions of cells according to manufacturer's protocol. For fresh tissues we proceeded to microRNAs extraction once a pathologist had confirmed the presence of at least the 70% of malignant cells, checking a snap frozen section.

RNAs from FFPE samples were extracted using RecoverAll Total Nucleic Acid Isolation kit (Ambion, Austin, TX, U.S.A.), according to manufacturer's instructions. The hematoxylin and eosin (H&E) sections were reviewed by a pathologist to select the more informative block. Four 20 µm-thick sections were cut, followed by one H&E control slide. The area selected for the analysis was marked on the control slide to ensure, whenever possible, greater than 90% content of glial cells (normal adjacent the tumor and epileptic specimens) or neoplastic cells (glioma samples).

Then the four 20 µm-thick sections were manually macro-dissected using a blade according to area selected on H&E and RNAs extraction was performed according to manufacturer's protocol.

Quality and quantity of RNAs extracted from both Fresh/Frozen and FFPE-dissected tissue were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and the Qubit fluorometer (Invitrogen, Carlsbad, CA, U.S.A.).

3.4 MicroRNAs analysis

3.4.1 Assay design

Nineteen microRNAs (miR-7, miR-9, miR-9*, miR10a, miR10b, miR-17, miR-20a, miR-21, miR-26a, miR-27a, miR-31, miR-34a, miR-101, miR-137, miR-182, miR-221, miR-222, miR-330, miR-519d) were chosen according to their role in cancer and previously published data in literature at beginning of the study [58-60, 62, 71, 74, 77-81, 91, 94-96, 99, 100, 113, 114]. MicroRNA103 and two small RNAs (RNU49 and U54) were used as endogenous controls.

MicroRNAs expression was analyzed with quantitative Real-Time PCR (qRT-PCR), using specific primers designed to recognize the mature microRNA sequences, according to miRBase database (http://microrna.sanger.ac.uk) (Table

3.1). Forward primers for qRT-PCR were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/).

Due to their short length and the high sequence similarity within microRNA families, a reliable and accurate quantification system for microRNA analysis was needed. For this reason LNA bases were introduced in each forward primer which allowed the design of short, very high specific, primers covering most of the mature microRNAs sequences. In fact the LNA methylene bridge (Figure 3.1A), causing a conformational change of the duplex, confers more rigidity to the structure ensuring a higher bond strength between primer and template sequence: once introduced in the positions where specificity and discrimination is needed, LNA improves mismatch discriminations.

Moreover, this conformational change increases the annealing temperature of duplex allowing the design of shorter primers. So we have designed each microRNA primer with 2 or 3 LNA substitutions (Table 3.1), spaced at least one nucleotide, assuring an average melting temperature of 60°C for each primer. To ensure a good discrimination between microRNAs different just for a single nucleotide (e.g. miR-10a and miR-10b, Table 3.1, Figure 3.1) a LNA nucleotide was introduced in correspondence of the discriminating base.

Universal reverse primer was provided by NCode miRNA first-strand cDNA synthesis and qRT-PCR Kit (Invitrogen, Carlsbad, CA, U.S.A.).

miRNA	Fw Primer Sequence used in this study
hsa-miR-7	TGGAA**G**ACTA**G**TGATTTTGTT
hsa-miR-9	TCTTTG**G**TTATCTAG**C**TGTATG
hsa-miR-9*	ATAAAG**C**TA**G**ATAA**C**CGAAAG
hsa-miR-10a	ACC**C**TGTAGA**T**CCGAATTTG
hsa-miR-10b	ACC**C**TGTAGA**A**CCGAATTTG
hsa-miR-17	CAAAGTGCTTA**C**A**G**TGCAG
hsa-miR-20a	CAAAGTGCTTA**T**A**G**TGCAG
hsa-miR-21	TAG**C**TTATCA**G**ACTGATGTTG
hsa-miR 26a	CAAGTAAT**C**CAGGATA**G**GC
hsa-miR-27a	TTCA**C**AGTGGCTAAGTTC**C**
hsa-miR 31	AGGCAA**G**ATGCTGG**C**ATA
hsa-miR-34a	TGG**C**AGTGT**C**TTAGCTG
hsa-miR-101	TACAGTA**C**TGT**G**ATAACTGAA
hsa-miR-137	TTATTG**C**TTAAGAATA**C**GCGT
hsa-miR-182	TTTGGCA**A**TGGTAGAA**C**TCAC
hsa-miR-221	GCTA**C**ATTGTCTG**C**TGGGTT
hsa-miR-222	GCTA**C**ATCTGG**C**TACTGG
hsa-miR-330	TCT**C**TGGGCCTGTGT**C**TTA
hsa-miR-519d	AAGTGC**C**TCC**C**TT**T**AGAGT
miRNA normalizers	
RNU49	CTGA**C**GAAGACTACT**C**CTGT
U54	GT**G**ATAATTTTATA**C**GCTATT**C**TGA
hsa-miR-103	CAGCATTGTA**C**AGGGCTAT**G**

Table 3.1. Forward primer sequences of analyzed microRNAs. LNA bases are underlined. Fw: forward. Hsa: Homo sapiens (human).

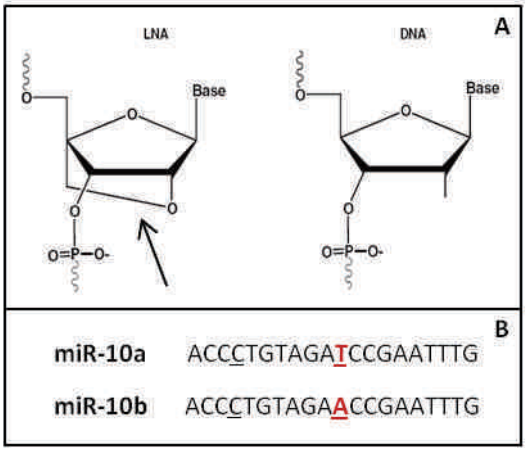


Figure 3.1. Example of microRNAs forward primers with LNA substitutions. A) LNA and DNA bases: the arrow indicated the 2'O-4'C Methylene bridge. B) Forward primers for microRNAs 10a and 10b which differ for only nucleotide, that is indicated in red. LNA substitutions are underlined.

3.4.2 Primer efficiency

To evaluate the efficiency of each primer the small RNAs fractions of 3 cell lines were extracted and pooled together: U87-MG (glioblastoma), MCF7 (breast cancer) and LNCaP (prostate cancer). Each primer was tested on several cDNA dilutions (1:1, 1:25, 1:50, 1:100) and a standard curve titration for each microRNA primer was obtained. We considered a good efficiency in the range from 90% to 110%. Moreover a pool of female DNA (Promega, Madison, WI, U.S.A.) was used as template to verify that each microRNA primer was not able to amplify DNA.

3.4.3 MicroRNAs expression analysis in brain samples

Both for Fresh/Frozen and FFPE samples, RNA extracted was retro-transcribed using the NCode miRNA Firststrand cDNA synthesis and qRT-PCR Kit protocol (Invitrogen, Carlsbad, CA, U.S.A.). Briefly cDNAs were obtained after a polyadenylation step and retrotranscriptions were performed using SuperScript III RT enzyme and a Universal RT Primer according to manufacturer's protocol. The qRT-PCR mixes were prepared using FastStart Taq Reagents Kit (Roche, Mannheim, Germany) and microRNAs expression was evaluated using a AB7000 machine (Applied Biosystem, Foster City, CA, USA) with the following program: 2 minutes at 50°C, 4 minutes at 95°C followed by 37 cycles for 30 seconds at

95°C, 30 seconds of annealing step at 60°C and 30 seconds at 72°C with fluorescence measurement. GelStar stain (Lonza Bioscience, Rockland, ME, USA) was used as Real-Time detector. No template control for each microRNA was included in the reaction plate. Each microRNA was run twice per each sample. Amplicons were run on a 3% agarose gel. FirstChoice®Human Brain Reference RNA (Ambion, Austin, TX, U.S.A.), considering that it was a pool of RNAs, it was analyzed three times (technical replicates).

3.4.4. Bioinformatics prediction of microRNAs targets

The experimentally validated targets of those microRNAs which resulted significantly deregulated in GBM profile, had been identified through several online tools: miRecords (http://mirecords.umn.edu/miRecords/) [40], miRTarBase (http://mirtarbase.mbc.nctu.edu.tw) [41] and miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk)[42]. In detail the last releases of miRecords (http://mirecords.umn.edu/miRecords/download.php, updated on November 25th, 2010) and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/download.php, "hsa MTI.xls" file, release 3.5 of November 1st, 2012) were downloaded and only microRNAs of interest were selected. While the list of selected microRNAs was uploaded on miRWalk "*Validated target(gene) of mirna search"* section (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/mirnatargetpub.html, updated on March 11th, 2011) to obtained the list of experimentally validated targets relative to each microRNAs of interest.

In particular we focused our attention only on those targets identified by all 3 prediction tools used. To analyze and grouped targets according to their molecular functions, biological process involvement and pathways classification, PANTHER web tool was used (http://www.pantherdb.org/).

3.5 MGMT methylation analysis

At least 50 ng of DNA were treated with bisulfite using the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A Methylation Sensitive Real Time qPCR using 3'-locked nucleic acid (LNA)

modified primers and beacon probes (MS-qLNAPCR, Table. 3.2) was performed [115]. The analysis was performed using FastStart Universal Probe Master with ROX (Roche Applied Science, Mannheim, Germany) on the AB7000 machine (Applied Biosystem, Foster City, CA, USA) with the following conditions: 95°C for 4 minutes, 60°C for 2 minutes, 72°C for 2 minutes, followed by 40 cycles for 20 seconds at 95°C, 45 seconds at 60°C with fluorescence detection, 30 seconds at 72°C.

	MGMT Methylated	MGMT UNMethylated
Primer Fw	5′-TTT**C**GACGTT**C**GTAGGTTTT**C**G**C**-3′	5′-TTTGTGTTT**T**GA**T**GTT**T**GTAGGTTTT**T**G**T**-3′
Beacon probe	5′-FAM-CCGGAGCGTAT**C**GTTTG**C**GA-TTTGGTGAGTGTGCTCCGG-BHQ1-3′	5′-FAM-CCGGTGCTGTAT**T**GTTTG**T**GATT-TGGTGAGTGTGCACCGG-BHQ1-3′
Primer Rv	5′-**G**CACTCTTCC**G**AAAAC**G**AAAC**G**-3′	5′-AACTCC**A**CACTCTTCC**A**AAAAC**A**AAAC**A**-3′

Table 3.2. Primers and beacon probes for MGMT-MSqLNAPCR: LNA nucleotides are underlined; CpG discriminatory LNA nucleotides are in bold.[115] Fw: forward; Rv: reverse.

3.6 IDH1 mutation analysis

We tested all tumor samples for *IDH1*-R132H mutation using Allele Specific Locked Nucleic Acid quantitative PCR (ASLNAqPCR), set up by our group for *KRAS* and *BRAF* analysis [116]. Briefly 15–50 ng of DNA extracted from FFPE samples, were amplified using the FastStart Universal Probe Master with ROX (Roche Applied Science, Mannheim, Germany) in separate Real Time reactions for each allele specific primer. Using Primer3 software (http://frodo.wi.mit.edu/primer3/), we designed allele specific primers to recognize the IDH1 wild type sequence or the R132H mutation. Both forward primers had been designed with 3'-locked nucleic acid (LNA) substitutions to improve mismatch discrimination (Table 3.3). The analysis was performed using FastStart Universal Probe Master with ROX (Roche Applied Science, Mannheim, Germany) on the AB7000 machine (Applied Biosystem, Foster City, CA, USA) with the following program: 2 minutes at 50°C, 4 minutes at 95°C followed by 37 cycles for 30 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C with

fluorescence measurement. GelStar stain (Lonza Bioscience, Rockland, ME, USA) was used for signal detection.

	IDH1 WT Allele	IDH1 R132H Allele
Primer Fw	5'-TTGATCCCCATAAGCATGAC - 3'	5'- GTGGCACGGTCTTCAGAGA -3'
Primer Rv	5'- TTGATCCCCATAAGCATGAT - 3'	5'- GTGGCACGGTCTTCAGAGA -3'

Table 3.3. Primers for IDH1- ASLNAqPCR: LNA nucleotides are underlined. WT: wild type; Fw: forward; Rv:reverse.

3.7 Statistical analysis

3.7.1 MicroRNAs analysis

DataAssist 2.0 Tool (Applied Biosystem, Foster City, CA, USA) was used to obtain expression values and fold-changes using the relative quantification and the $2^{-\Delta\Delta Ct}$ method [117]. The unsupervised hierarchical clustering analysis (Pearson Correlation, average linkage) were performed using the same statistical tool. GraphPad Prism 5.0 tool was used for correlation analysis between groups applying Spearman correlation test or Wilcoxon paired test for Fresh/Frozen and FFPE-dissected sample pairs analysis. For comparing the expression levels of each miRNA between different groups, Kruskal-Wallis and Mann-Whitney tests were used. Gaussian distribution was evaluated by Shapiro-Wilk Test. In microRNAs analysis in general we adopted a fold change cut-off of twofold: we considered as downregulated a microRNA with a ratio <-2.0, while a microRNA with a ratio $\geq$ 2.0 was considered as upregulated. Level of significance was $p<0.05$ for all the statistical analysis.

3.7.2 MicroRNAs target analysis

A Statistical overrepresentation test [118] was performed using PANTHER web tool (http://www.pantherdb.org/tools/uploadFiles.jsp). The target genes list was compared to a reference list to statistically determine over- or under-representation of PANTHER classification categories. This binomial statistical test was applied to analyze PANTHER molecular functions, biological processes

and PANTHER pathway classifications. P-values were calculated with Bonferroni correction and a p-value cutoff of 0.05 was considered to estimate if a particular PANTHER category was over- or under- represented in a significant statistical manner than would be expected by chance (indicated by the Expected Values).

3.7.3 MGMT Methylation analysis and IDH1 mutation analysis

The relative methylated or mutant allele copy number was quantified during the exponential phase of Real Time qPCR using the ΔΔCT method [117].

Chapter 4:

Results

4.1 Sample collection and preservation: under vacuum treatment versus RNA later fixation

The best protocol was optimized for sample collection and conservation, avoiding or minimizing the nucleic acid degradation.

The fixation in RNA later solution, before freezing in liquid nitrogen, and the under vacuum treatment of tissues in plastic bags were performed in parallel.

All possible times needed for the transport of the specimens from surgical units to our lab, were tested. We have compared samples maintained under vacuum for 0-6-24-48 hours with the same samples quick-frozen in liquid nitrogen after the RNA Later incubation, immediately after surgical removal.

Under vacuum treatment was proved to be not suitable for these purposes because some samples were smashed up due to the mechanic stress of vacuum machine and, most of all, the RNA quality was not preserved: RNA was more degraded after under vacuum treatment than when samples were fixed in RNA later and a RNA later fixation after the arrival to our lab did not preserved RNA quality (Figure 4.1).

As shown in Figure 4.1, RNA quality was not preserved after under vacuum treatment even at time zero and RNA was totally degraded after 48h. After RNA later fixation (even stored for 48h) a quite good quality of RNA was obtained. For this reasons, we have chosen and adopted the RNA later protocol for sample collection and storage.

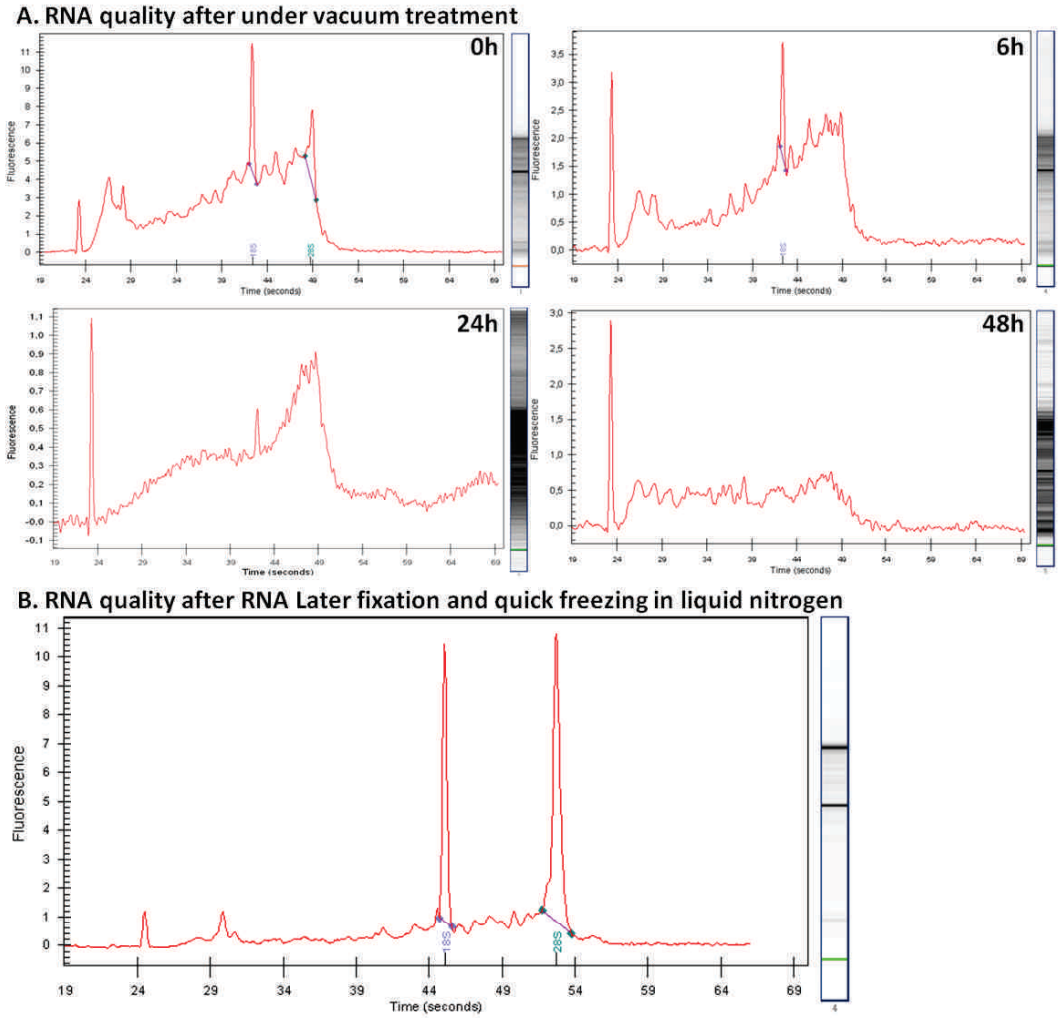


Figure 4.1. RNA quality analysis through Agilent 2100 Bioanalyzer platform. A) RNA quality analysis of samples underwent to under vacuum treatment for 0-6-24-48 hours. The RNA was partially degraded even just at time zero and totally degraded after 48 hours. B) A better RNA quality was observed analyzing a sample quick-frozen in liquid nitrogen after fixation step with RNA Later solution. The BioAnalyzer software showed an electropherogram and gel-like image, that represented two peaks related to the subunit 18S and 28S. The area under the curve is proportional to RNA concentration of the sample.

4.2 Comparison of microRNAs expression between Fresh/Frozen and FFPE-dissected GBM specimens

To set up our method for the analysis of microRNAs expression in GBM samples, we performed qRT-PCR starting from microRNAs extracted both from Fresh/Frozen and FFPE specimens. In this way the feasibility of our technique on both type of specimens was tested and possible discrepancies in microRNAs expression values between the two sample types were analyzed. We could analyzed microRNAs expression of 30 GBM samples pairs (Table 4.1), available both as Fresh/Frozen (FF) and FFPE-dissected (FD) specimens.

For Fresh/Frozen samples a preliminary 4µm-thick snap frozen section was made and checked to verify the presence of the tumor. We could not perform microRNA extraction starting from snap frozen slides because the necessity of several sections should lead to an excessive sample manipulation, increasing the risk of RNA degradation. We proceeded with microRNAs extractions starting from pieces of tissue of 20-50 mg in weight, notwithstanding we were not able to quantify if the percentage of malignant cells, estimated on snap frozen control slide, was maintained in the whole sample that we used for analysis. Even if the choice of Fresh/Frozen tissue as starting material ensure a good quality of RNA, it was not possible to know the real cellular composition of the specimen: in fact some non-neoplastic cells (e.g. lymphocytes, neurons, normal glial cells, endothelial cells, etc) could be present in the sample used for the analysis and this possibility could altered the microRNAs expression results.

For this reason in parallel microRNAs from FFPE specimens were also extracted after macro-dissection of four 20µm-thick slides according to the tumor area selected on an haematoxylin and eosin. In this way a good enrichment (>90%) in neoplastic cells was ensured avoiding normal cells contaminations.

4.2.1 GBM Fresh/Frozen and FFPE-dissected sample pairs: MGMT and IDH1 status

Out of 30 GBM cases analyzed, 14 (46.7%) resulted methylated for MGMT promoter (Table 4.1). All cases resulted to be wild-type for IDH1, supporting the evidence of "primary GBM"[119] (Table 4.1).

Case N°	Code	Age	Sex	MGMT status	IDH1 Status	Case N°	Code	Age	Sex	MGMT status	IDH1 Status
1	BO0072	51	M	UMET	WT	16	BO0652	69	M	UMET	WT
2	BO0158	70	F	MET	WT	17	BO0674	75	F	MET	WT
3	BO0160	42	F	MET	WT	18	BO0858	74	F	UMET	WT
4	BO0162	68	F	UMET	WT	19	BO0902	68	F	MET	WT
5	BO0337	69	M	UMET	WT	20	BO0923	56	M	MET	WT
6	BO0363	65	F	UMET	WT	21	BO0956	69	M	UMET	WT
7	BO0364	71	F	MET	WT	22	BO0968	45	M	UMET	WT
8	BO0375	65	F	UMET	WT	23	BO1081	75	F	MET	WT
9	BO0386	71	F	MET	WT	24	BO1238	60	M	UMET	WT
10	BO0530	41	F	MET	WT	25	BO1278	75	M	MET	WT
11	BO0556	61	F	UMET	WT	26	BO1353	50	M	UMET	WT
12	BO0558	55	F	MET	WT	27	BO1378	49	F	MET	WT
13	BO0582	70	M	UMET	WT	28	BO1412	75	M	UMET	WT
14	BO0583	60	M	UMET	WT	29	BO1477	48	M	MET	WT
15	BO0584	77	M	MET	WT	30	BO1487	67	F	UMET	WT

Table 4.1. Glioblastoma cases available both as Fresh/Frozen and FFPE-dissected specimens. MGMT methylation and IDH1 mutation status are also indicated. MET: methylated; UMET: unmethylated; WT: wild type.

4.2.2 Unsupervised hierarchical clustering analysis

Unsupervised hierarchical clustering analysis, based on the variation of expression of each microRNA across the specimens analyzed, showed that the matched samples generally did not cluster together (Figure 4.2A). Figure 4.2B shows the unsupervised hierarchical clustering analysis of Fresh/Frozen sample normalized on five normal brain specimens. The same sample analyzed as FFPE-dissected specimens showed a better separation between tumor and normal group (Figure 4.2C – see Appendix A for higher magnification).

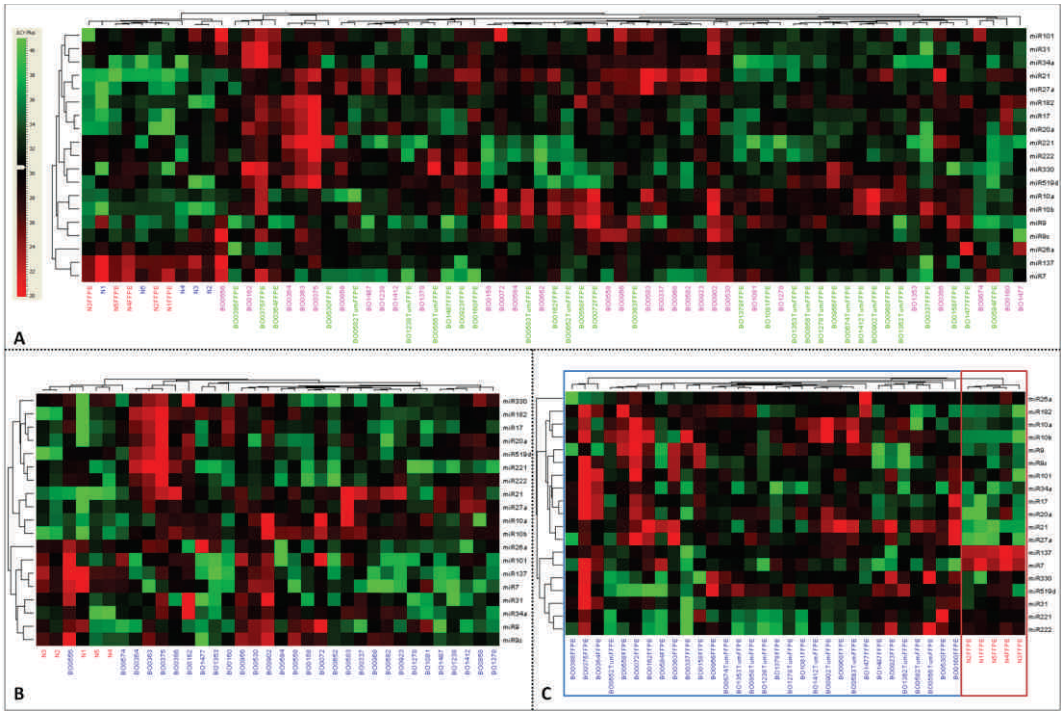


Figure 4.2. Unsupervised hierarchical clustering analysis of 30 Fresh/Frozen and FFPE-dissected GBM for the 19 microRNAs analyzed. A) Clustering analysis of the 30 paired samples, both tumors and normal brain specimens. FFPE-dissected GBM samples are written in green, Fresh/Frozen GBM in pink, Fresh/Frozen normal brains in blue and FFPE control samples in red. B) Clustering analysis of the 30 Fresh/Frozen GBM samples: GBM group is written in blue and normal brain samples in red. C) Clustering analysis of the 30 FFPE GBM samples: GBM group is represented in blue and normal brain samples in red. Blue square highlights GBM group and red square the control group. N: normal sample; BO-: anonymous code for GBM samples.

4.2.3 Correlation between Fresh/Frozen and FFPE-dissected groups for microRNAs expression analysis

According to the Shapiro Test, the distribution for 30 Fresh/Frozen (FF) and FFPE-dissected (FD) GBM sample pairs was not normal ($p<0.001$). As a consequence, non-parametric statistical tests were used.

Good correlation ($r=0.7916$, $p<0.0001$) was obtained when expression levels of each microRNA between Fresh/Frozen and FFPE-dissected groups were compared (Spearman correlation test, Figure 4.3).

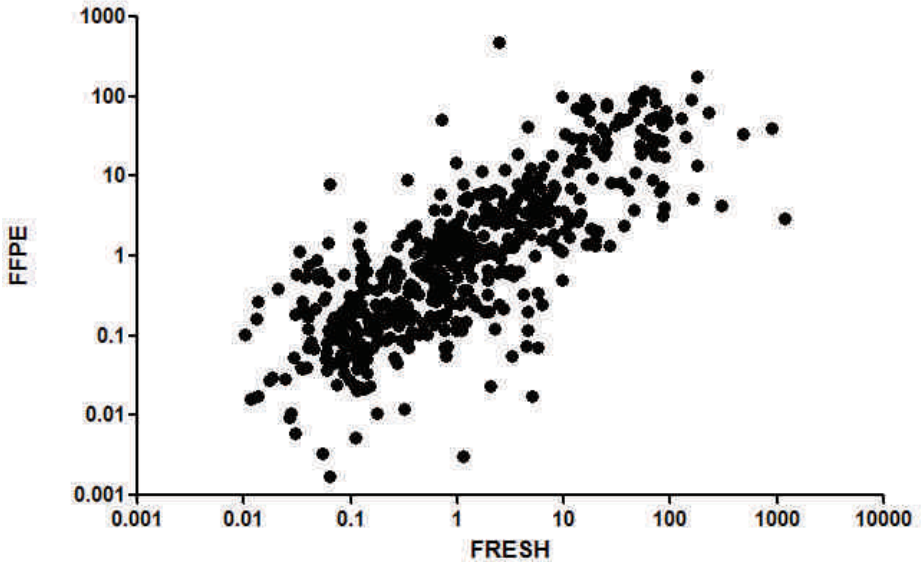
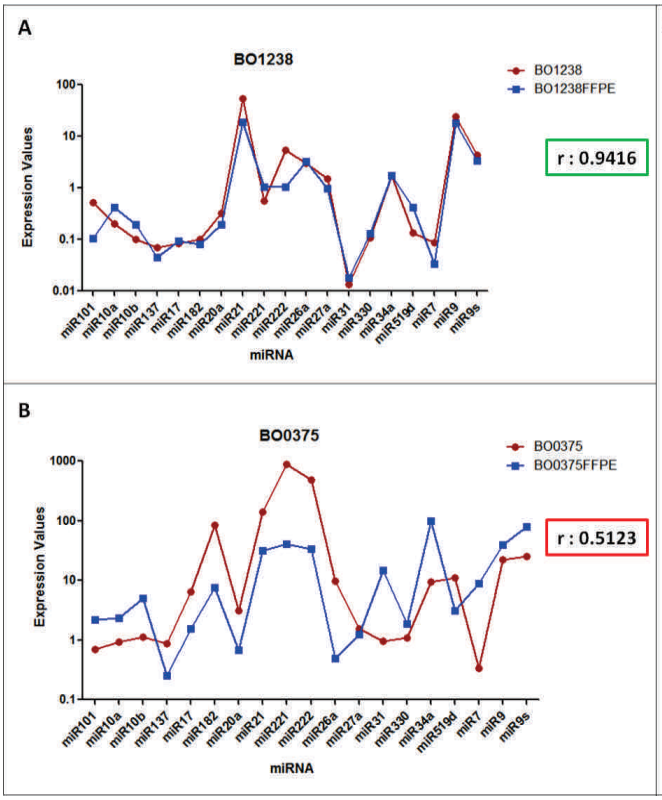


Figure 4.3. Spearman correlation scatter plot between Fresh/Frozen and FFPE-dissected sample groups. FRESH: Fresh/Frozen sample group; FFPE: FFPE-dissected sample group.

Considering each specimens pair, good correlation values were obtained (ranged from 0.5123 to 0.9421) between the microRNAs expression levels (Figure 4.4C). In particular this comparison showed a good correlation coefficient ($r>0.65$) in 25 out of the 30 sample pairs analyzed (Figure 4.4C).

We further compared microRNAs expression levels of all samples coupled in two groups (Fresh/Frozen versus FFPE-dissected) using Wilcoxon paired t-test and not significant differences were observed ($p=0.1845$). Analyzing samples comparing each Fresh/Frozen specimen with the corresponding FFPE-dissected one, 8 pairs out of 30 showed statistically significant differences (p-value<0.05, Table 4.2).

Moreover performing an Anova test with Bonferroni correction, significant differences between the two groups were observed in the following 4 microRNAs: miR-9*, miR-21, miR-221 and miR-222.



C

Sample	Spearman coefficient	p-value
BO0072	0,9421	<0,0001
BO0158	0,8386	<0,0001
BO0160	0,5842	=0,0006
BO0162	0,5158	=0,019
BO0337	0,8495	<0,0001
BO0363	0,7158	=0,0003
BO0364	0,5386	=0,0016
BO0375	0,5123	=0,0125
BO0386	0,7105	=0,0003
BO0530	0,5201	=0,0023
BO0556	0,5368	=0,0017
BO0558	0,9158	<0,0001
BO0582	0,8018	<0,0001
BO0583	0.817	<0,0001
BO0584	0,8474	<0,0001
BO0652	0,9386	<0,0001
BO0674	0,5737	=0,0008
BO0858	0,7842	<0,0001
BO0902	0,5772	=0,0007
BO0923	0,8789	<0,0001
BO0956	0,5614	=0,001
BO0968	0,8934	<0,0001
BO1081	0,9123	<0,0001
BO1238	0,9416	<0,0001
BO1278	0,8596	<0,0001
BO1353	0,8404	<0,0001
BO1378	0,8509	<0,0001
BO1412	0,8416	<0,0001
BO1477	0,5863	=0,0006
BO1487	0,8018	<0,0001

Figure 4.4. Spearman correlation test analyzing Fresh/Frozen and FFPE-dissected sample pairs. A) An example of a good correlation between paired specimens with a Spearman correlation value of 0.9416. B) An example of a not good correlation between paired specimens with a Spearman correlation value of 0.5123. C) Table showed all Spearman correlation values obtained comparing the 30 GBM sample pairs.

Case N°	Sample	p-value	Significance	Case N°	Sample	p-value	Significance
1	BO0072	0,1230	ns	**16**	BO0652	0,4094	Ns
2	BO0158	0,0382	*	**17**	BO0674	0,0559	Ns
3	BO0160	0,0094	**	**18**	BO0858	0,5328	Ns
4	BO0162	0,7022	ns	**19**	BO0902	0,0007	***
5	BO0337	0,0613	ns	**20**	BO0923	0,6435	Ns
6	BO0363	0,0421	*	**21**	BO0956	0,0464	*
7	BO0364	0,2197	ns	**22**	BO0968	0,4566	Ns
8	BO0375	0,6726	ns	**23**	BO1081	0,9839	Ns
9	BO0386	0,0018	**	**24**	BO1238	0,2197	Ns
10	BO0530	0,3869	Ns	**25**	BO1278	0,0119	*
11	BO0556	0,1313	Ns	**26**	BO1353	0,6435	Ns
12	BO0558	0,9839	Ns	**27**	BO1378	0,7022	Ns
13	BO0582	0,7323	Ns	**28**	BO1412	0,9839	Ns
14	BO0583	0,1650	Ns	**29**	BO1477	0,0800	Ns
15	BO0584	0,0313	*	**30**	BO1487	0,7628	Ns

Table 4.2. Wilcoxon paired test results for the 30 GBM pairs. Eight samples out of 30 analyzed showed significant differences considering a p-value cutoff of 0.05. ns: not significant difference was observed. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$.

4.2.4 Differences in microRNAs expression between Fresh/Frozen and FFPE-dissected specimens

In spite of the good correlation values obtained between Fresh/Frozen and FFPE-dissected groups, we verified if microRNAs profiles of the two groups were comparable or not.

We first analyzed microRNAs profile of each Fresh/Frozen GBM versus its corresponding FFPE-dissected one and then we calculated the median fold-change of each microRNA.

Considering a twofold fold change cut-off, we observed that 3 microRNAs (miR-137, miR-20a and miR-21) were differentially expressed in Fresh/Frozen samples if compared with FFPE-dissected ones (FF/FD ratio >2.0), while the remnant microRNAs did not shown significantly differences in expression values between FF and FD samples (Figure 4.5).

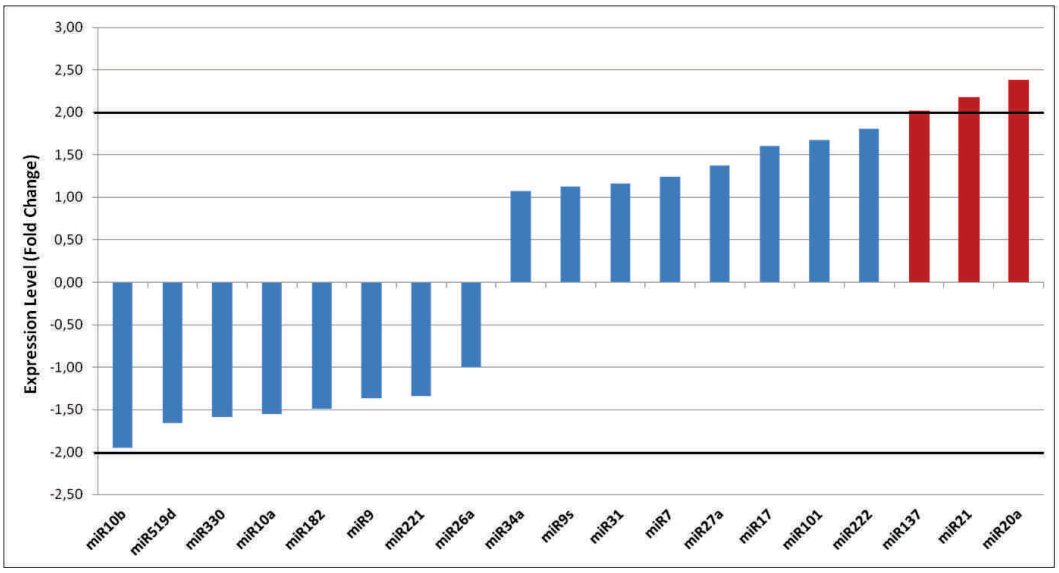


Figure 4.5. Median microRNAs expression levels of Fresh/Frozen samples compared with FFPE-dissected ones. The two bold lines on the graph highlight the fold change cut-off adopted: a microRNA was considered upregulated when it showed a FF/FD ratio >2 or downregulated when FF/FD ratio <-2. MicroRNAs which resulted differentially expressed in Fresh/Frozen samples in comparison with FFPE-dissected ones, are in red.

4.2.5 Correlation between Fresh/Frozen, FFPE-dissected and FFPE-not dissected samples for microRNAs expression analysis

In the 5 cases showing a Spearman coefficient <0.65 (Figure 4.4C) we investigated if the low correlation values could be due to enrichment in neoplastic cells in dissected samples.

We analyzed the microRNAs profiles of these 5 samples starting from not dissected FFPE sections. In 1 out of the 5 cases in analysis (Case#4: BO162), the H&E revealed that there was not present not-neoplastic tissue adjacent the neoplastic area (Table 4.3).

Comparing Fresh/Frozen and FFPE-not dissected samples we obtained a good Spearman correlation value, which increased up the cut-off of 0.65, in 3 out of 4 cases analyzed (Table 4.3).

Case N°	Code	R (FF vs FD)	R (FF vs FND)	Composition of not-dissected FFPE samples	
				Neoplastic cells (%)	Not-neoplastic cells (%)
4	BO162	0,51	NP	98	2
7	BO364	0,63	0,70	50	50
8	BO375	0,51	0,38	75	25
10	BO530	0,62	0,81	70	30
11	BO556	0,63	0,89	40	60

Table 4.3. Spearman correlation values analyzing Fresh/Frozen, FFPE-dissected and FFPE-not dissected sample pairs. FF: Fresh/Frozen; FD: FFPE-dissected; FND: FFPE-not dissected; NP: Not Performed.

4.3 Non-neoplastic brain references: normal adjacent the tumor, epileptic tissues and commercial brain RNA

Among the several non-neoplastic controls commonly adopted as reference in GBM microRNAs analysis, we had the possibility to compare the following three: normal area adjacent the tumor (N-Ad); one of the available commercial normal brain RNA (Ref), purchased from Ambion; tissue removed in epileptic patients (EP).

One out of 15 N-Ad in analysis did not give sufficient quantity of microRNAs to be used in the analysis.

Shapiro Test reveled that the distribution for N-Ad, Ref and EP groups was not a Gaussian ($p<0.001$). In according to this result, we used non-parametric statistical tests.

4.3.1 Correlation among normal area adjacent the tumor (N-Ad), commercial normal brain RNA reference (Ref) and epileptic tissue (EP) groups

We analyzed the expression of 19 miRNAs in all 3 references and all Spearman correlation values obtained were above 0.65, with the best correlation between Epileptic tissue and the commercial reference groups (Table 4.4).

	N-Ad	Ref	Ep
N-Ad	/	0.724	0.702
Ref	0.724	/	**0.848**
Ep	0.702	**0.848**	/

Table 4.4. Spearman correlation values between three groups ($p<0.0001$). Ref: Ambion commercial reference; Ep: epileptic group; N-Ad: Normal Adjacent group.

Analyzing the median expression values of each microRNA in the three different groups, significant differences were observed (comparing the 3 groups together with Kruskal Wallis test, $p<0.05$) in 9 microRNAs: miR-7, miR-9, miR-10a, miR-10b, miR-26a, miR-27a, miR-31, miR-137, and miR-182 (Table 4.5).

Moreover considering groups in pairs (Mann-Whitney test), the significant differences obtained with Kruskal Wallis test were confirmed and in addition statistical significant differences even in microRNA-101 (between N-Ad and Ref) and microRNA-519d (between N-Ad and EP) were observed (Figure 4.6 – see Appendix B for higher magnification).

miRNAs	Median Expression Values			p-value*
	Normal Adjacent Tumor ± Median Error	*Ambion Brain Reference ± Median Error*	*Epileptic Tissue ± Median Error*	
miR-7	1.441 ± 0.336	2.255 ± 1.515	4.517 ± 0.660	p<0.01
miR-9	15.898± 3.508	45.191 ± 9.489	24.512 ± 3.744	p<0.05
miR-9*	1.388± 0.876	4.767 ± 1.504	2.118 ± 0.572	NS
miR-10a	0.422± 0.249	0.706 ± 0.332	0.207 ± 0.116	p<0.05
miR-10b	0.356± 0.203	0.258 ± 0.193	0.086 ± 0.118	p<0.05
miR-17	0.031± 0.038	0.059 ± 0.003	0.051 ± 0.015	NS
miR-20a	0.039± 0.024	0.094 ± 0.001	0.049 ± 0.020	NS
miR-21	0.910± 0.814	2.473 ± 0.313	0.974 ± 0.668	NS
miR-26a	2.699± 0.698	17.851 ± 1.429	4.649 ± 0.779	p<0.01
miR-27a	0.541± 0.166	0.402 ± 0.036	0.201 ± 0.075	p<0.05
miR-31	0.077± 0.072	0.366 ± 0.091	0.062 ± 0.017	p<0.05
miR-34a	0.833± 0.512	0.933 ± 0.355	0.759 ± 0.212	NS
miR-101	0.254± 0.085	0.632 ± 0.053	0.444 ± 0.171	NS
miR-137	0.316± 0.099	0.663 ± 0.226	1.042 ± 0.298	p<0.01
miR-182	0.232± 0.131	0.263 ± 0.091	0.093 ± 0.029	p<0.01
miR-221	3.753± 0.701	2.914 ± 0.999	5.018 ± 0.907	NS
miR-222	19.427± 9.525	6.466 ± 2.036	10.655 ± 6.789	NS
miR-330	0.691± 0.217	0.309 ± 0.053	0.371 ± 0.290	NS
miR-519d	0.644± 0.259	0.742 ± 0.248	0.309 ± 0.119	NS

Table 4.5. Median expression values obtained in the three different groups. *p-values were obtained using Kruskal-Wallis test.

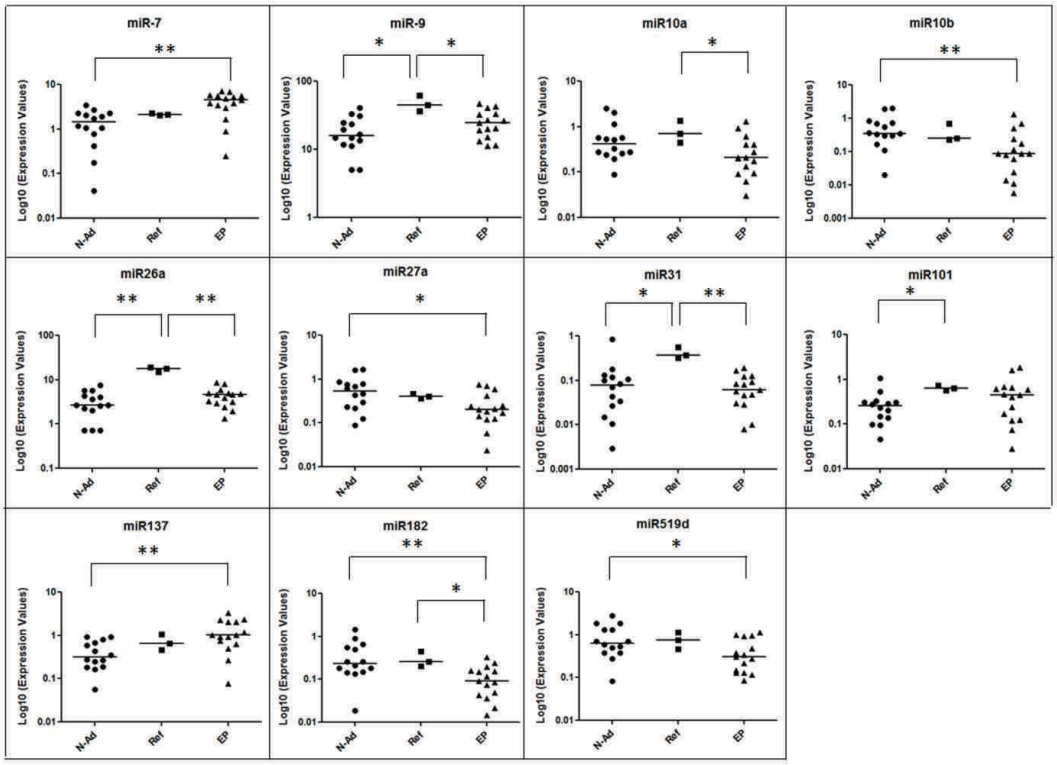


Figure 4.6. Differences in microRNAs expression among three non-neoplastic references. Scatter plots show microRNAs significantly different among the three groups. * p<0.05, ** p<0.01 according to Mann-Whitney test. N-Ad: Normal adjacent the tumor; Ref: Ambion Commercial reference; EP: epileptic tissue.

4.4 GBM microRNAs profile

Within the PERNO project, up to 50 glioblastoma specimens for molecular analysis were collected.

In the case BO0375 no residual material was available for further analysis.

4.4.1 Glioblastoma cases: MGMT and IDH1 status

Out of 50 GBM cases analyzed, 22 (44%) resulted methylated for MGMT promoter (Table 4.6). All cases resulted to be wild-type for IDH1, supporting the evidence of "primary GBM"[119] (Table 4.6).

Case N°	Code	Age	Sex	MGMT Status	IDH1 status	Case N°	Code	Age	Sex	MGMT status	IDH1 status
1	BO0072	51	M	UMET	WT	**26**	BO1353	50	M	UMET	WT
2	BO0158	70	F	MET	WT	**27**	BO1362	50	M	MET	WT
3	BO0160	42	F	MET	WT	**28**	BO1378	49	F	MET	WT
4	BO0162	68	F	UMET	WT	**29**	BO1412	75	M	UMET	WT
5	BO0337	69	M	UMET	WT	**30**	BO1477	48	M	MET	WT
6	BO0363	65	F	UMET	WT	**31**	BO1487	67	F	UMET	WT
7	BO0364	71	F	MET	WT	**32**	CE0226	58	M	UMET	WT
8	BO0386	71	F	MET	WT	**33**	CE0328	64	F	UMET	WT
9	BO0487	78	M	UMET	WT	**34**	CE0332	51	F	UMET	WT
10	BO0530	41	F	MET	WT	**35**	CE0478	66	F	NA	NA
11	BO0556	61	F	UMET	WT	**36**	CE0636	60	F	UMET	WT
12	BO0558	55	F	MET	WT	**37**	CE0691	41	M	MET	WT
13	BO0582	70	M	UMET	WT	**38**	CE0667	59	M	UMET	WT
14	BO0583	60	M	UMET	WT	**39**	CE0693	63	M	MET	WT
15	BO0584	77	M	MET	WT	**40**	CE1049	53	M	MET	WT
16	BO0652	69	M	UMET	WT	**41**	CE1158	74	M	UMET	WT
17	BO0674	75	F	MET	WT	**42**	CE1159	47	F	UMET	WT
18	BO0858	74	F	UMET	WT	**43**	CE1286	53	M	UMET	WT
19	BO0902	68	F	MET	WT	**44**	CE1310	63	M	UMET	WT
20	BO0923	56	M	MET	WT	**45**	FA0571	58	M	UMET	WT
21	BO0956	69	M	UMET	WT	**46**	FO0559	56	F	MET	WT
22	BO0968	45	M	UMET	WT	**47**	FO1173	72	F	MET	WT
23	BO1081	75	F	MET	WT	**48**	RA1209	72	M	UMET	WT
24	BO1238	60	M	UMET	WT	**49**	RN0565	60	M	MET	WT
25	BO1278	75	M	MET	WT	**50**	RN1423	73	F	MET	WT

Table 4.6. Glioblastoma cases collected by the PERNO project. MGMT methylation and IDH1 mutation status are also indicated. MET: methylated; UMET: unmethylated; NA:not avaible; WT: wild type.

4.4.2 Definition of a GBM microRNAs profile

Considering the differences in microRNAs expression observed among the three non-neoplastic brain references previously analyzed (Results section 4.3), we wondered if the choice of one reference could influence GBM microRNAs profile definition. For this reason, we analyzed GBM microRNAs expression matching the 50 GBM cases on all 3 non-neoplastic references. A cut-off of 2 fold-change had been considered to identify microRNAs differentially expressed. As expected, the median expression values of the 19 microRNAs in GBM group were different according to non-neoplastic reference adopted as control (Table 4.7). In order to define a GBM microRNAs profile, a microRNA was considered as dysregulated when it showed the same dysregulated status in at least 2 out of the 3 profiles. The final GBM profile is shown in Table 4.8.

MiR-137, miR-31 and miR-7 were downregulated independently by the non-neoplastic reference chosen (Tables 4.7 and 4.8, Figure 4.8). In addition miR-9, miR-26a, miR-101, miR-222 and miR-330 were downregulated in at least two out of three profiles obtained (Tables 4.7 and 4.8, Figure 4.8). Interesting all microRNAs with a FC< -2 were also downregulated in at least 50% of analyzed glioblastomas (Table 4.7).

Only 3 microRNAs resulted upregulated in at least 2 out of the 3 profiles obtained: in particular microRNA-21 was upregulated (FC>2) independently by the reference (Tables 4.7 and 4.8, Figure 4.7) while miR-10b and miR-27a were upregulated only considering Ambion reference and Epileptic group as controls (Tables 4.7 and 4.8, Figure 4.7). As seen before, also in this case all miRNAs with a FC> 2 were upregulated in at least 50% of analyzed tumor cases (Table 4.7). Four microRNAs (miR-9*, miR-17, miR-20a, miR34a) showed expression levels that were within twofold cut-off independently by the non-neoplastic control used and were considered not deregulated. MiR-10a, miR-182, miR-221 and miR-519d showed a dysregulated status in only 1 profile out of 3 analyzed (considering normal adjacent to the tumor as control for miR-519d or Epileptic group for miR-10a, miR-182 and miR-221) (Table 4.7). For this reason we excluded these microRNAs from GBM profile.

miRNA	GBM Group vs Ambion Reference				GBM Group vs Epileptic Group				GBM Group vs Normal Adjacent Group			
	Up[a]	Down[b]	Median FC[c] ± Median Error	Status	Up[a]	Down[b]	Median FC[c] ± Median Error	Status	Up[a]	Down[b]	Median FC[c] ± Median Error	Status
miR7	0	48	-23.641 ± 0,033	DOWN	0	48	-50.633 ± 0,016	DOWN	0	45	-15.723 ± 0,050	DOWN
miR9	4	28	-2.260 ± 0,139	DOWN	4	25	-2.144 ± 0,147	DOWN	14	17	1.043 ± 0,328	=
miR9*	10	11	-1.052 ± 0,406	=	10	10	-1.056 ± 0,405	=	13	11	1.047 ± 0,448	=
miR10a	12	7	1.254 ± 0,280	=	34	3	3.040 ± 0,680	UP	18	5	1.513 ± 0,338	=
miR10b	33	3	2.537 ± 0,736	UP	40	1	4.273 ± 1,240	UP	20	8	1.637 ± 0,475	=
miR17	15	11	1.320 ± 0,637	=	15	11	1.301 ± 0,628	=	11	16	1.075 ± 0,519	=
miR20a	9	11	-1.133 ± 0,178	=	18	6	1.228 ± 0,247	=	18	5	1.317 ± 0,265	=
miR21	44	0	6.506 ± 1,615	UP	48	0	10.077 ± 2,501	UP	45	0	6.832 ± 1,696	UP
miR26a	1	48	-8.587 ± 0,087	DOWN	2	28	-2.229 ± 0,337	DOWN	5	17	-1.331 ± 0,564	=
miR27a	28	0	2.095 ± 0,363	UP	31	0	2.237 ± 0,388	UP	8	8	-1.007 ± 0,172	=
miR31	1	46	-15.221 ± 0,084	DOWN	6	29	-2.811 ± 0,457	DOWN	4	38	-5.417 ± 0,237	DOWN
miR34a	14	6	1.126 ± 0,615	=	14	5	1.221 ± 0,667	=	7	24	-1.903 ± 0,287	=
miR101	2	35	-2.550 ± 0,113	DOWN	2	37	-2.661 ± 0,107	DOWN	6	7	-1.153 ± 0,248	=
miR137	0	49	-9.074 ± 0,020	DOWN	0	50	-16.051 ± 0,011	DOWN	0	45	-5.408 ± 0,034	DOWN
miR182	24	8	1.924 ± 1,066	=	39	1	4.944 ± 2,738	UP	14	11	-1.049 ± 0,528	=
miR221	5	14	-1.246 ± 1,319	=	3	31	-2.393 ± 1,728	DOWN	3	16	-1.408 ± 2,941	=
miR222	4	24	-1.945 ± 0,398	=	1	48	-12.747 ± 0,061	DOWN	1	47	-10.040 ± 0,077	DOWN
miR330	6	12	-1.507 ± 0,236	=	1	41	-5.168 ± 0,069	DOWN	1	41	-4.187 ± 0,085	DOWN
miR519d	5	22	-1.575 ± 0,152	=	13	18	1.004 ± 0,241	=	2	25	-2.134 ± 0,113	DOWN

Table 4.7. MicroRNAs expression profile in GBM Group obtained using the three different non neoplastic references. [a] Number of GBM upregulated for the corresponding miRNA; [b] Number of GBM downregulated for the corresponding miRNA; [c] We assumed a cutoff of 2 fold change to consider microRNA as deregulated. FC: Fold-change; UP: upregulated; DOWN: downregulated; =: not deregulated

Status	miRNA	Non-neoplastic reference	Median fold Change of GBM Group versus		
			Ref*	Ep*	N-Ad*
Upregulated	miR-10b	Ref, Ep	2.537 (33/50)	4.273 (40/50)	ND
	miR-21	Ref, Ep, N-Ad	6.506 (44/50)	10.077 (48/50)	6.832 (45/50)
	miR-27a	Ref, Ep	2.095 (28/50)	2.237 (31/50)	ND
Downregulated	miR-7	Ref, Ep, N-Ad	-23.641 (48/50)	-50.633 (48/50)	-15.723 (45/50)
	miR-9	Ref, Ep	-2.261 (28/50)	-2.144 (25/50)	ND
	miR-26a	Ref, Ep	-8.587 (48/50)	-2.229 (28/50)	ND
	miR-31	Ref, Ep, N-Ad	-15.221 (46/50)	-2.811 (29/50)	-5.417 (38/50)
	miR-101	Ref, Ep	-2.551 (35/50)	-2.661 (37/50)	ND
	miR-137	Ref, Ep, N-Ad	-9.074 (49/50)	-16.051 (50/50)	-5.408 (45/50)
	miR-222	Ep, N-Ad	ND	-12.474 (48/50)	-10.041 (47/50)
	miR-330	Ep, N-Ad	ND	-5.168 (41/50)	-4.187 (41/50)

Table 4.8. Final GBM microRNAs profile. MicroRNAs up- or downregulated in GBM in at least 2 profiles obtained with different non-neoplastic references are shown**. *** Number of GBMs which shared the same deregulation status are indicated between brackets. Ref: Ambion commercial reference; Ep: epileptic group; N-Ad: Normal Adjacent group; ND: not deregulated.

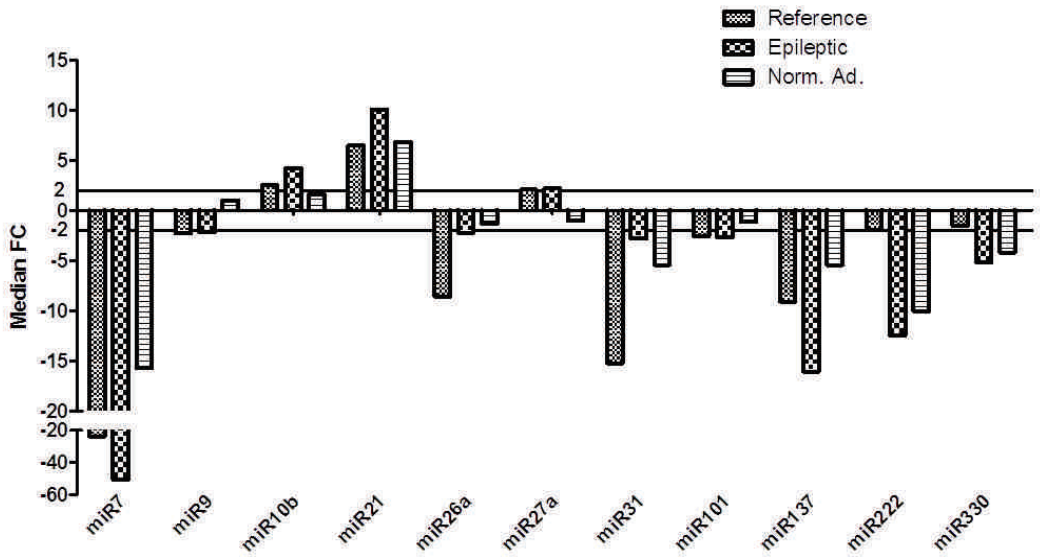


Figure 4.7. Final GBM microRNAs profile considering microRNAs up- or downregulated in at least 2 profiles obtained with different non-neoplastic references. The two bold lines on the graph highlight the cut-off of 2 fold-change. Reference: Ambion Commercial Reference; Epileptic: Epileptic Group; Norm. Ad.: Normal adjacent the tumor group; FC: fold change.

4.4.3 Correlation between GBM microRNAs profiles obtained using the three non-neoplastic references

Spearman correlation test was used to evaluate the correlation between the 3 GBM microRNAs profiles obtained with the three non-neoplastic references (Table 4.9).

The best correlation value was observed between profiles obtained using Ambion commercial reference and Epileptic group as controls (Table 4.9). Moreover these two profiles shared the higher number of microRNAs with the same regulation status (Table 4.7 and Table 4.8).

	N-Ad	Ref	Ep
N-Ad	/	0.7596 (11/19)	0.8421 (10/19)
Ref	0.7596 (11/19)	/	0.8970 (14/19)
Ep	0.8421 (10/19)	0.8970 (14/19)	/

Table 4.9. Spearman correlation values between microRNAs profiles obtained using the three different non-neoplastic references ($p<0.0001$)**.** The number of miRNAs which shared the same deregulation status are indicated between brackets. Ref: Ambion commercial reference; Ep: epileptic group; N-Ad: Normal Adjacent group.

4.4.4 Comparison between MGMT methylated and un-methylated GBM samples

Unsupervised hierarchical clustering analysis was performed to compare MGMT methylated (MET) and un-methylated (UMET) GBM samples, depending on the variation of expression of each microRNA across the specimens analyzed. Only the 11 microRNAs demonstrated deregulated in GBM profile were considered (Figure 4.8 – see Appendix C for higher magnification).

Figure 4.8 shows the unsupervised hierarchical clustering analysis of MET-GBM and UMET-GBM groups normalized on three normal brain specimens (Ambion Commercial Reference in Figure 4.8A, Normal adjacent the tumor in Figure 4.8B and Epileptic tissues in Figure 4.8C). It was not observed a clear clusterization between the two groups as regard the methylation status, independently by the reference. This result indicated that there was not a significant correlation between methylation status of MGMT promoter and microRNA expression analysis, considering this subset of microRNAs.

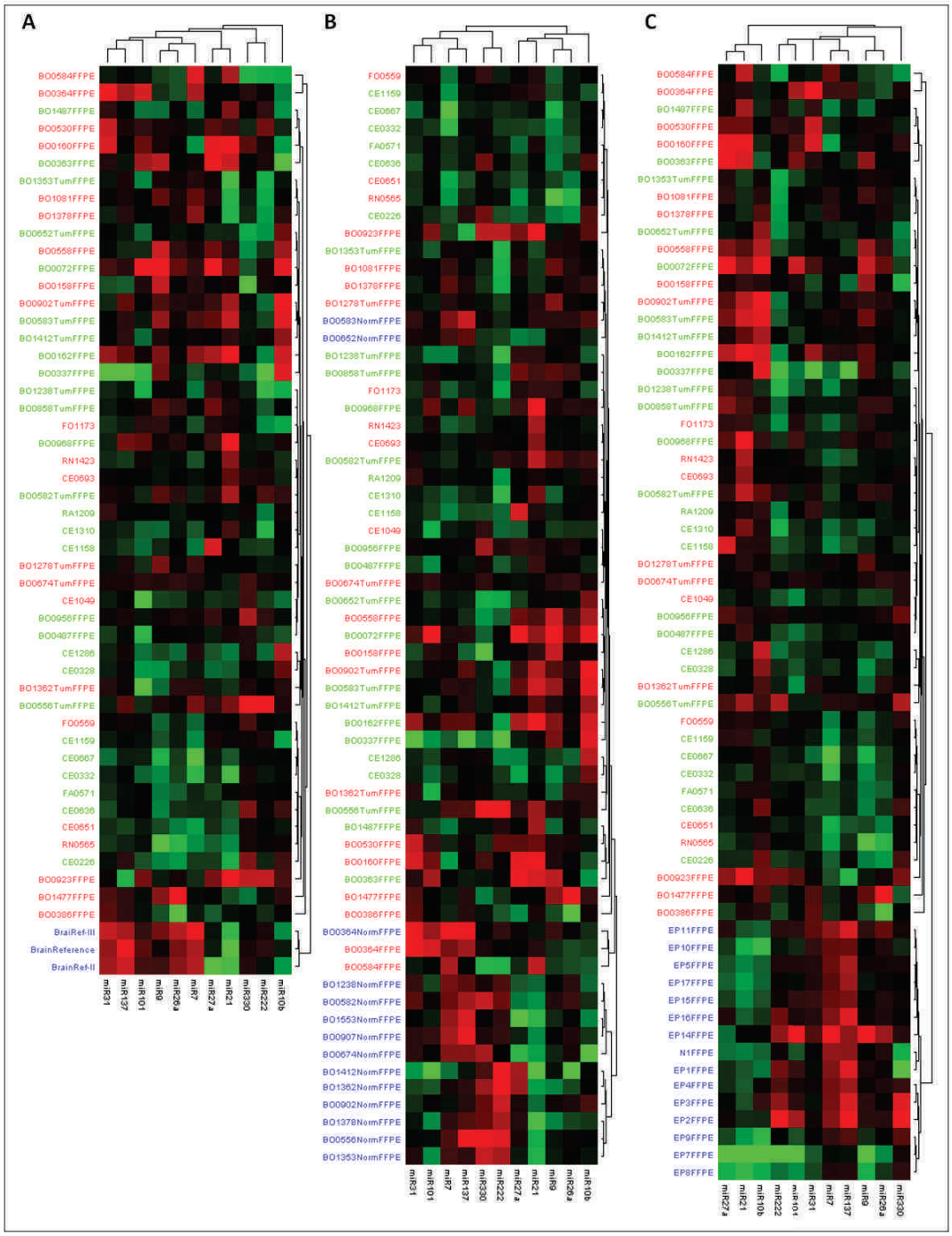


Figure 4.8. Unsupervised hierarchical clustering analysis of MET-GBM and UMET-GBM for the 11 selected microRNAs. Clustering analysis of MET-GBM and UM-GBM samples was performed using, Ambion Commercial Reference (A), Normal adjacent the tumor (B) and Epileptic tissues (C). MET-GBM samples are written in red, UMET-GBM in green, non-neoplastic control samples in blue.

4.5 Putative targets analysis

4.5.1 Research of validated targets of deregulated microRNAs

Considering only the 11 microRNAs (miR-7, miR-9, miR-10b, miR-21, miR-26a, miR-27a, miR-31, miR-101, miR-137, miR-222, miR-330) with a significant deregulation in GBMs, we decided to investigate their predicted targets. Taking into account that the functional analysis of possible mRNA targets of deregulated microRNAs was not a purpose of this project, we restricted our analysis to the research of their known targets, experimentally validated in previous studies. To do this we used online bioinformatics tools like miRecords, miRTarBase and miRWalk. We listed and analyzed all targets found and, as expected, we found different numbers of targets, some shared by all three tools used.

In particular considering the 11 microRNAs all together, we found 102 validated targets on miRecords and 219 on miRTarBase, reminding that both tools consider manually curated experimental interactions based on literature surveying (Figure 4.9A). On the other hand we found a higher number of targets on miRWalk (710 targets, Figure 4.9A), which is based on automated text-mining search. Among them only 56 were shared by all analysis tools as shown in Figure 4.9A. In Figure 4.9B are reported targets relative to each microRNA. In Table 4.10 targets found through all 3 bioinformatics tools, relative to each microRNA, are shown.

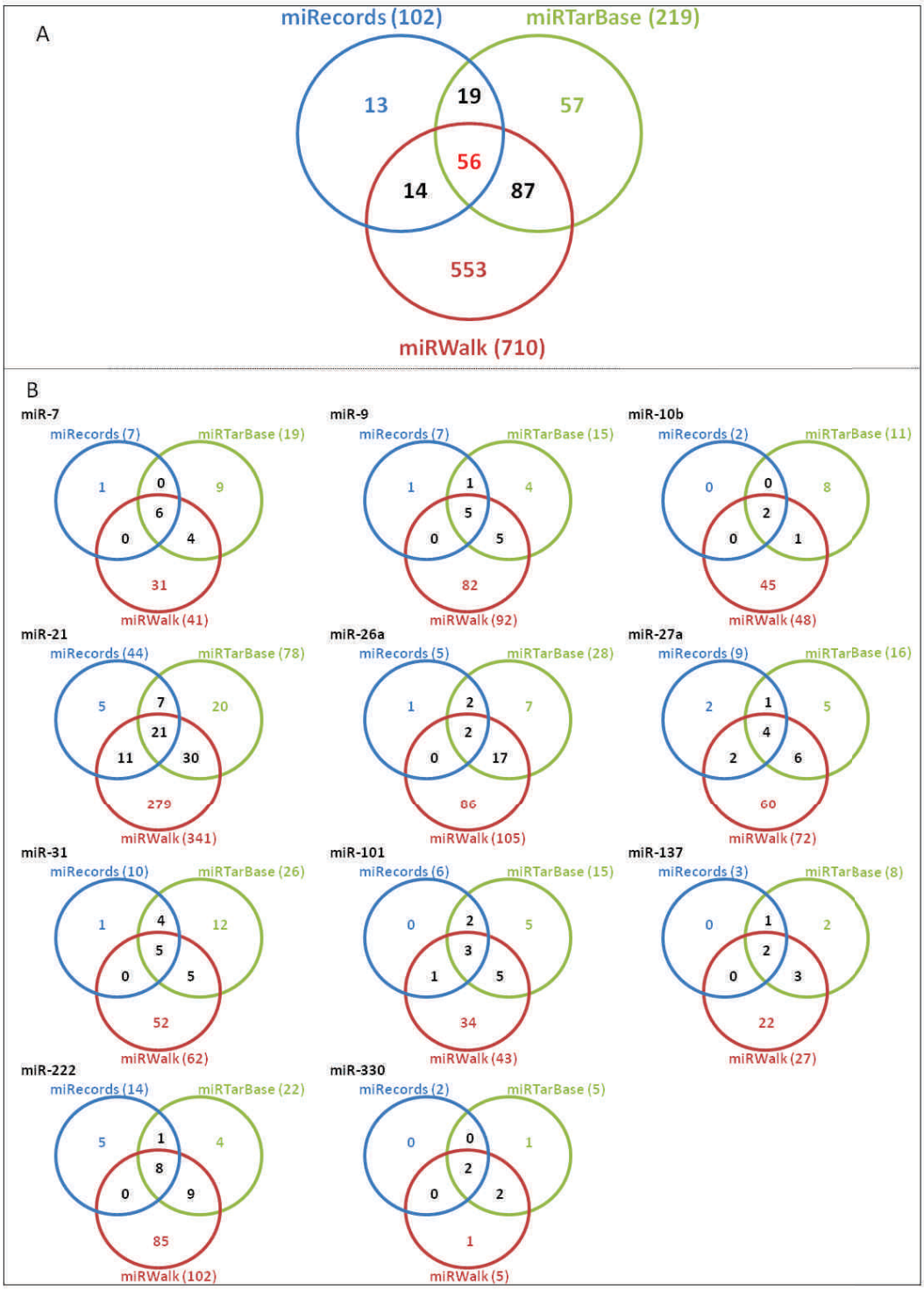


Figure 4.9. Validated targets of the microRNAs GBM profile. A) Targets found through 3 different tools considering 11 selected microRNAs all together**.** B) Targets found through 3 different tools considering microRNA individually. Between brackets is indicated the number of targets found using each single tool, while inside circles the numbers of shared targets are indicated.

miRNA	Validated targets found through all 3 bioinformatics tools used
miR-7	EGFR, IRS1, IRS2, PAK1, RAF1, SNCA
miR-9	BACE1, CDH1, NFKB1, PRDM1, REST
miR-10b	HOXD10, KLF4
miR-21	APAF1, BMPR2, BTG2, CDC25A, E2F1, HNRNPK, JAG1, LRRFIP1, MARCKS, MTAP, NFIB, PDCD4, PTEN, RASA1, RASGRP1, RECK, SOX5, TGFB1, TGFBR2, TIMP3, TPM1
miR-26a	EZH2, SMAD1
miR-27a	FOXO1, PHB, Sp3, Sp4
miR-31	ITGA5, LATS2, PPP2R2A, RDX, RHOA
miR-101	ATXN1, EZH2, MCLN1
miR-137	CDK6, NCOA2
miR-222	CDKN1B, CDKN1C, ESR1, KIT, MMP1, PPP2R2A, PTEN, SOD2
miR-330	E2F1, VEGFA

Table 4.10. MicroRNAs targets shared by miRecords, miRTarBase and miRWalk.

4.5.2 Preliminary analysis of microRNAs targets: pathways involvement, molecular function and biological process information

To perform a preliminary analysis of putative pathways controlled and modified by these deregulated microRNAs, PANTHER database (http://www.pantherdb.org) was used

In particular the 56 gene targets, reported by all previously used tools (Figure 4.9A), were further investigated. This list of gene targets was uploaded on PANTHER web tool and pathways, molecular function and biological process information were downloaded. From the initial gene list of 56 targets, PANTHER was unable to map the gene KLF4, for this reason all further analysis were done on 55 gene targets list. In Figure 4.10 PANTHER pathways analysis is reported: the 55 genes uploaded had been clusterized depending on their pathways involvement. From this preliminary analysis these targets seemed to be involved for example in Insulin/IGF pathway-MAPKK/MAP kinase cascade, angiogenesis, gonadotropin-releasing hormone receptor (GNRHR), Insulin/IGF-PKB and interleukin pathways as shown in Figure 4.10.

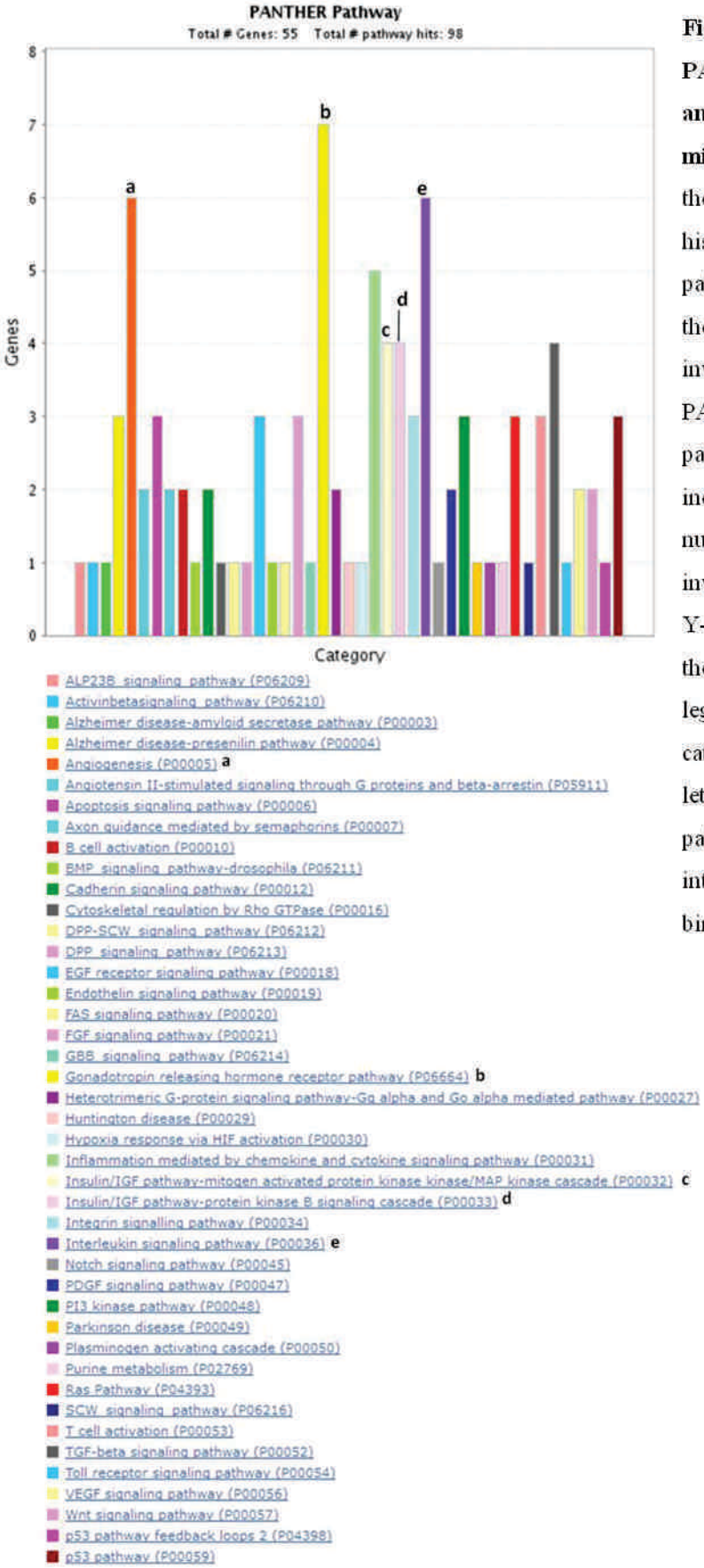


Figure 4.10. PANTHER pathways analysis of 55 miRNAs targets. On the top is reported an histogram of found pathways in which these 55 genes are involved depending on PANTHER database: pathway category is indicated in X-axis, the number of gene involved is reported in Y-axis. On the bottom the PANTHER color legend of pathways categories: is indicated: letters highlight pathways of main interest depending on binomial statistical test.

In Figure 4.11 the Molecular Function analysis of 55 microRNAs targets is shown: 55 genes were classified in Molecular Function categories depending on PANTHER database. The most represented was the Binding Molecular Function Category (GO:0005488), in which 35 genes, out of 55 analyzed, were classified. Among them for example 19 target genes were classified as members of "Nucleic Acid Binding Molecular Function" Class (GO:0003676) (Figure 4.11B).

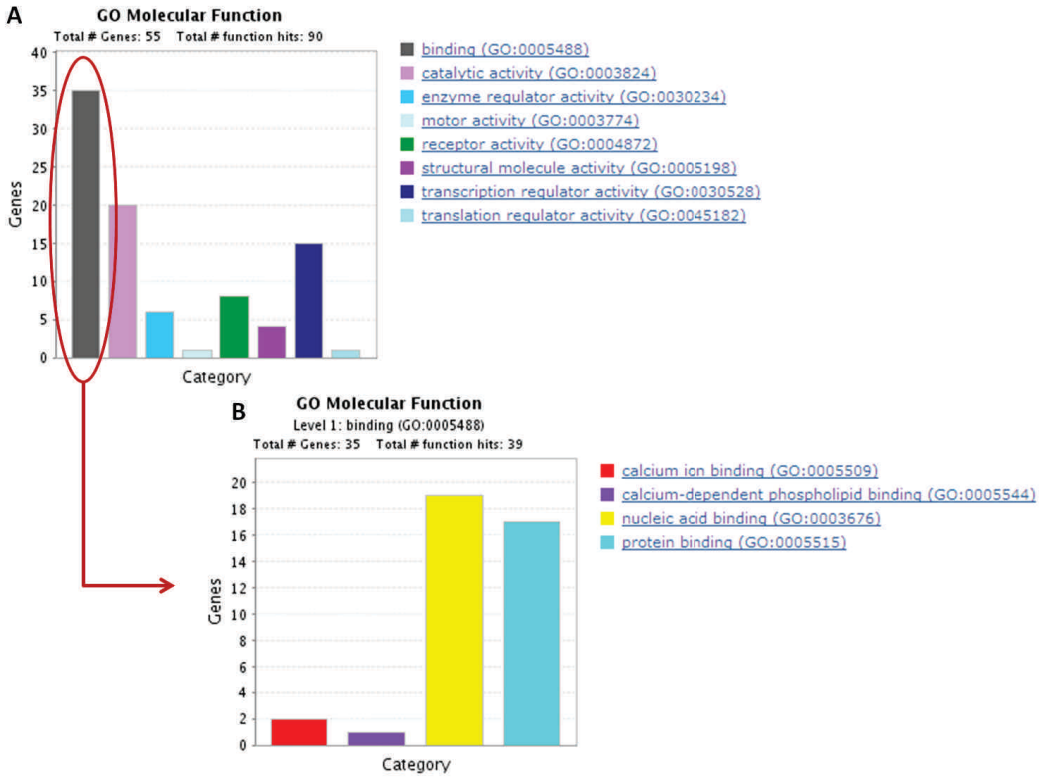


Figure 4.11. PANTHER Molecular Function analysis of 55 microRNAs targets. A) The histogram represents the Molecular Function classes in which these 55 genes are classified depending on PANTHER database: GO Molecular Function category is indicated in X-axis, the number of member genes is reported in Y-axis. B) The detail of Binding Molecular Function class (GO:0005488), in which 35 genes out of 55 analyzed are classified depending on PANTHER database, is presented: for example 19/35 genes are members of Nucleic Acid Binding Molecular Function Class (GO:0003676). GO:-, Gene Ontology number.

In Figure 4.12 the Biological Process analysis of 55 microRNAs targets is reported: 55 genes were classified in Biological Process categories depending on PANTHER database. The most represented was the Cellular Process Category (GO:0009987), in which 36 genes, out of 55 analyzed, were classified. Among them for example 27 target genes were classified as members of Cell Communication Biological Process Class (GO:0007154) (Figure 4.12B).

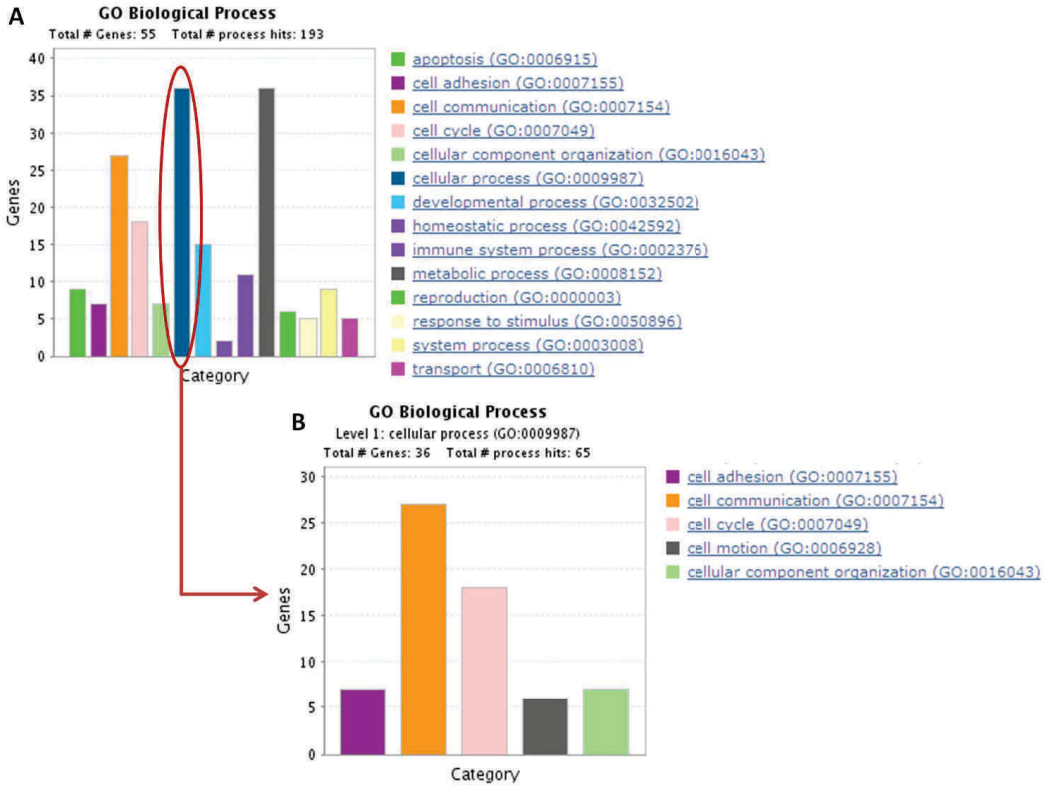


Figure 4.12. PANTHER Biological Process analysis of 55 microRNAs targets. A) The histogram represents the Biological Process classes in which these 55 genes are classified depending on PANTHER database: GO Biological Process category is indicated in X-axis, the number of member genes is reported in Y-axis. B) The detail of Cellular Process class (GO:0009987), in which 36 genes out of 55 analyzed are classified depending on PANTHER database, is presented: for example 27/36 genes are members of Cell Communication Biological Process class (GO:0007154). GO:-, Gene Ontology number.

4.5.3 Statistical overrepresentation test

The PANTHER binomial statistic tool [118] was used to determine if there was a significant statistical over- or under- representation of 55 target genes in comparison with a reference list (in this case the default Homo Sapiens Whole Genome list was selected). The statistical significance was represented by p-values: a small p-value ($p<0.05$) indicated that the number of observed genes in each category analyzed (pathway, molecular function or biological process for example) was not occurred by chance (randomly). We considered a p-value cutoff of 0.05. P-values reported in Table 4.11 were obtained using the Bonferroni correction for multiple testing. In Table 4.11 pathways, molecular function classes and biological processes interested by 55 gene targets were listed: in particular only those that had shown significant p-values had been presented and in all cases a statically significant over-representation of number of target genes per category was observed.

In detail, in comparison to the expected value, which represented the number of genes we would expect in our target genes list for a particular PANTHER category based on the reference list, we observed for all significant categories more genes in target genes list than expected. So we obtained a significant overrepresentation for all categories listed in Table 4.11 and the significant p-values indicated that these results were non-random and potentially interesting.

PANTHER classification category	Number of genes			p-value	% of gene list in the category[d]
	Reference List[a]	Target list[b]	Expected[c]		
Pathways					
Interleukin signaling pathway	152	6	0.42	7.19E-04	11,76%
Insulin/IGF pathway- MAPKK/MAPK cascade	38	4	0.10	7.28E-04	7,84%
Gonadotropin releasing hormone receptor pathway	282	7	0.78	2.20E-03	13,73%
Angiogenesis	186	6	0.51	2.25E-03	11,76%
Insulin/IGF pathway- PKB signaling cascade	77	4	0.21	1.13E-02	7,84%
Molecular functions					
binding	6916	35	19.02	1.57E-03	68,63%
transmembrane receptor protein kinase activity	152	5	0.42	9.58E-03	9,80%
protein kinase activity	523	8	1.44	1.32E-02	15,69%
Biological Processes					
cellular process	6400	36	17.60	6.33E-05	70,59%
cell cycle	1867	18	5.13	2.37E-04	35,29%
signal transduction	4293	27	11.81	9.31E-04	52,94%
cell communication	4504	27	12.39	2.38E-03	52,94%
intracellular signaling cascade	1613	15	4.44	3.56E-03	29,41%
protein amino acid phosphorylation	713	10	1.96	3.80E-03	19,61%
transmembrane receptor protein serine/threonine kinase signaling pathway	98	4	0.27	2.75E-02	7,84%
induction of apoptosis	440	7	1.21	3.43E-02	13,73%

Table 4.11. PANTHER overrepresentation analysis. [a] Number of genes in the reference list that map to this particular PANTHER classification category; [b] Number of genes in the target genes list that map to this particular PANTHER classification category, [c] Expected value is the number of genes that we could expect in target genes list for this PANTHER category based on the reference list; [d] In the last column is indicated the percentage of target genes list which represented each category. P-values are determined by binomial statics with Bonferroni correction: a p-value cutoff of 0,05 was considered.

4.6 MicroRNAs expression analysis in gliomas of grade I, II and III

4.6.1 Grade I, II and III glioma cases

Once set up the method for microRNAs analysis on FFPE samples and once the GBM microRNAs profile was defined, we thought to evaluate if there were some differences in microRNAs expression associated with different grade of malignancy. We were able to retrieve 45 cases of brain tumors of grade lower than the IV (Table 4.12).

In particular cases of gliomas of grade I, II and III were randomly selected from the archives of the Anatomic Pathology of Bellaria Hospital.

Fifteen cases per each grade were selected for microRNAs analysis (Table 4.12).

MicroRNAs fractions were extracted after macro-dissection of four FFPE 20µm-thick slides following the tumor area selected on a haematoxylin and eosin by a pathologist. Each case was analyzed for the same panel of microRNAs described before.

Grade I Gliomas				Grade II Gliomas				Grade III Gliomas			
Code	Sex	Age	Histological Diagnosis	Code	Sex	Age	Histological Diagnosis	Code	Sex	Age	Histological Diagnosis
GI-3	M	2	Pilocytic Astrocytoma	GII-23	M	49	Papyllary Ependymoma	GIII-47	M	54	Anaplastic Ependymoma
GI-4	M	15	Pilocytic Astrocytoma	GII-24	M	28	Ependymoma	GIII-49	M	31	Anaplastic Ependymoma
GI-5	F	17	Pilocytic Astrocytoma	GII-25	M	27	Oligodendroglioma	GIII-50	F	73	Anaplastic Oligodendroglioma
GI-6	F	23	Pilocytic Astrocytoma	GII-28	F	52	Oligodendroglioma	GIII-51	M	68	Anaplastic Oligodendroglioma
GI-7	M	24	Ganglioglioma	GII-29	M	55	Oligodendroglioma	GIII-52	M	30	Anaplastic Oligodendroglioma
GI-9	M	35	Ganglioglioma	GII-30	M	52	Oligodendroglioma	GIII-53	M	49	Anaplastic Oligodendroglioma
GI-11	F	27	Ganglioglioma	GII-31	M	58	Oligodendroglioma	GIII-54	F	46	Anaplastic Oligodendroglioma
GI-13	F	14	Ganglioglioma	GII-33	F	36	Oligodendroglioma	GIII-55	F	43	Anaplastic Oligodendroglioma
GI-14	F	14	Ganglioglioma	GII-34	M	63	Oligodendroglioma	GIII-56	F	50	Anaplastic Oligodendroglioma
GI-15	F	28	Ganglioglioma	GII-36	F	24	Pleomorphic Xanthoastrocytoma	GIII-57	M	42	Anaplastic Astrocytoma
GI-16	F	32	Ganglioglioma	GII-37	M	74	Astrocytoma	GIII-58	M	74	Anaplastic Astrocytoma
GI-17	F	31	Ganglioglioma	GII-41	M	21	Neurocytoma	GIII-59	M	64	Anaplastic Astrocytoma
GI-19	F	8	Ganglioglioma	GII-42	M	23	Neurocytoma	GIII-61	F	52	Anaplastic Oligoastrocytomas
GI-20	M	17	Ganglioglioma	GII-43	M	52	Neurocytoma	GIII-62	M	44	Anaplastic Oligoastrocytomas
GI-21	M	25	Ganglioglioma	GII-45	F	28	Neurocytoma	GIII-63	M	31	Anaplastic Oligoastrocytomas

Table 4.12. Cases of grade I, II and III gliomas. For each case code number, sex, age and histological diagnosis are indicated.

4.6.2 MicroRNAs expression analysis of grade I, II and III glioma cases

We analyzed all cases, starting from FFPE specimens, for the same microRNAs panel using the Ambion Commercial Reference and the Epileptic Group as controls (Tables 4.13 and 4.14).

In detail we focused our attention on the 14 microRNAs that had shown the same deregulation status in GBM expression profiles obtained using the commercial reference or epileptic tissues as non-neoplastic controls (Tables 4.7 and 4.8). We considered only these two GBM profiles because commercial reference and Epileptic group were the same references adopted for grade I, II and III brain tumor microRNAs analysis (Table 4.13).

miRNA	Grade IV vs Ref/EP	Grade I vs Ref/EP	Grade II vs Ref/EP	Grade III vs Ref/EP
miR7	DOWN	DOWN	DOWN	DOWN
miR9	DOWN	=	=	=
miR9*	=	=	=	=
miR10b	UP	DOWN	DOWN/= [a]	UP
miR17	=	=	=	=
miR20a	=	=	=	=
miR21	UP	UP	=	=/UP [a]
miR26a	DOWN	DOWN/=[a]	DOWN/= [a]	DOWN/= [a]
miR27a	UP	UP	=	=
miR31	DOWN	DOWN/= [a]	DOWN/= [a]	DOWN/= [a]
miR34a	=	UP	UP	=
miR101	DOWN	=	=	DOWN
miR137	DOWN	DOWN	DOWN	DOWN
miR519d	=	=	DOWN	DOWN

Table 4.13. MicroRNAs expression profiles obtained versus Ambion Commercial Reference/Epileptic Group. We considered a microRNA as deregulated when it reached the cutoff of 2 fold change and it showed the same deregulation status in at least the 50% of cases. [a] Cases in which the results obtained using the 2 references were not the same are reported as results on Commercial Refrence/results on Epileptic tissues. Ref: Ambion Commercial Reference; EP: Epileptic Group; UP: upregulated; DOWN: downregulated; =: not deregulated.

miRNA	GI Group vs Ambion Reference		GI Group vs Epileptic Group		GII Group vs Ambion Reference		GII Group vs Epileptic Group		GIII Group vs Ambion Reference		GIII Group vs Epileptic Group	
	Median Fc[a] ± Median Error	Status[b]	Median Fc[a] ± Median Error	Status[b]	Median Fc[a] ± Median Error	Status[b]	Median Fc[a] ± Median Error	Status[b]	Median Fc[a] ± Median Error	Status[b]	Median Fc[a] ± Median Error	Status[b]
miR7	-13,158 ± 0,182	DOWN (9/15)	-66,667 ± 0,084	DOWN (13/15)	-4,662 ± 0,408	DOWN (11/15)	-9,990 ± 0,190	DOWN (12/15)	-15,748 ± 0,018	DOWN (15/15)	-33,784 ± 0,009	DOWN (15/15)
miR9	-1,283 ± 0,337	=	-1,217 ± 0,356	=	-1,036 ± 0,226	=	1,018 ± 0,238	=	1,382 ± 0,323	=	1,457 ± 0,341	=
miR9s	1,159 ± 1,343	=	1,155 ± 1,339	=	1,377 ± 0,281	=	1,372 ± 0,280	=	1,420 ± 0,297	=	1,416 ± 0,296	=
miR10a	-1,713 ± 1,343	=	1,416 ± 0,680	=	-1,940 ± 1,986	=	1,250 ± 0,815	=	-1,737 ± 0,301	=	1,396 ± 0,730	=
miR10b	-3,676 ± 0,160	DOWN (8/15)	-2,182 ± 0,270	DOWN (8/15)	-2,744 ± 1,129	DOWN (8/15)	-1,629 ± 0,271	=	2,026 ± 0,722	UP (8/15)	3,413 ± 1,216	UP (9/15)
miR17	1,083 ± 0,257	=	1,068 ± 0,254	=	1,298 ± 0,253	=	1,279 ± 0,249	=	1,300 ± 0,360	=	1,282 ± 0,355	=
miR20a	-1,598 ± 0,209	=	-1,148 ± 0,291	=	-1,195 ± 0,081	=	1,165 ± 0,112	=	-1,540 ± 0,276	=	-1,107 ± 0,384	=
miR21	5,164 ± 1,381	UP (11/15)	7,999 ± 0,139	UP (12/15)	1,169 ± 0,335	=	1,810 ± 0,518	=	1,472 ± 0,997	=	2,280 ± 1,544	UP (9/15)
miR26a	-3,458 ± 0,085	DOWN (12/15)	1,114 ± 0,326	=	-3,839 ± 0,069	DOWN (13/15)	1,004 ± 0,265	=	-4,437 ± 0,551	DOWN (12/15)	-1,152 ± 0,121	=
miR27a	2,515 ± 0,611	UP (9/15)	2,686 ± 0,652	UP (9/15)	1,029 ± 0,145	=	1,100 ± 0,155	=	1,365 ± 0,375	=	1,458 ± 0,401	=
miR31	-3,195 ± 0,179	DOWN (10/15)	1,696 ± 0,970	=	-8,058 ± 1,617	DOWN (13/15)	-1,487 ± 0,760	=	-10,799 ± 0,018	DOWN (15/15)	-1,992 ± 0,097	=
miR34a	3,468 ± 1,839	UP (11/15)	3,763 ± 0,995	UP (12/15)	2,099 ± 1,590	UP (9/15)	2,277 ± 1,895	UP (9/15)	-1,086 ± 0,554	=	-1,001 ± 0,601	=
miR101	-1,152 ± 0,414	=	-1,203 ± 0,397	=	-1,355 ± 0,294	=	-1,414 ± 0,282	=	-2,299 ± 0,083	DOWN (9/15)	-2,400 ± 0,080	DOWN (9/15)
miR137	-2,892 ± 0,167	DOWN (10/15)	-5,123 ± 0,094	DOWN (12/15)	-5,139 ± 0,459	DOWN (10/15)	-9,099 ± 0,259	DOWN (10/15)	-14,881 ± 0,032	DOWN (15/15)	-26,316 ± 0,018	DOWN (15/15)
miR182	-1,786 ± 1,480	=	1,439 ± 0,803	=	-2,475 ± 0,631	DOWN (9/15)	1,038 ± 1,621	=	-1,251 ± 0,843	=	2,053 ± 0,305	UP (8/15)
miR221	1,496 ± 0,529	=	1,496 ± 0,276	=	-2,072 ± 0,749	DOWN (9/15)	-3,979 ± 0,390	DOWN (12/15)	-5,163 ± 0,104	DOWN (13/15)	-9,911 ± 0,054	DOWN (14/15)
miR222	1,559 ± 0,324	=	-4,202 ± 0,049	DOWN (14/15)	-2,991 ± 0,710	DOWN (8/15)	-19,608 ± 0,108	DOWN (14/15)	-6,439 ± 0,055	DOWN (14/15)	-42,194 ± 0,008	DOWN (14/15)
miR330	-1,963 ± 0,689	=	-6,729 ± 0,201	DOWN (12/15)	-3,043 ± 0,200	DOWN (10/15)	-10,438 ± 0,058	DOWN (14/15)	-7,831 ± 0,070	DOWN (13/15)	-26,882 ± 0,020	DOWN (15/15)
miR519d	-1,766 ± 0,135	=	-1,117 ± 0,537	=	-10,204 ± 0,356	DOWN (11/15)	-6,456 ± 0,563	DOWN (9/15)	-22,472 ± 0,113	DOWN (14/15)	-14,205 ± 0,178	DOWN (12/15)

Table 4.14. MicroRNAs expression profile in gliomas of grade I, II and III. [a] We assumed a cutoff of 2 fold change to consider microRNA as deregulated. [b] Between brackets the number of cases up- or downregulated for the corresponding microRNA is indicated. FC: Fold-change; UP: upregulated; DOWN: downregulated; =: not deregulated.

Considering these 3 profiles in comparison with the GBM one (grade IV previously obtained) (Table 4.8 and Table 4.13), some interesting differences were noticed among groups as regard microRNAs expression status. In particular we focused the attention on microRNAs which have showed different regulation status among the 4 glioma groups, excluding miR-9*, miR-17 and miR-20a because they resulted not deregulated in all analyzed profiles (Tables 4.13 and 4.14).

4.6.3 Unsupervised hierarchical clustering analysis of glioma groups

An unsupervised hierarchical clustering analysis, based on the variation of expression of those 14 microRNAs (Table 4.13) across the 4 groups of specimens analyzed, showed that there was not a clear separation between different grade gliomas (Figure 4.13 – see Appendix D for higher magnification), both using Ambion Commercial Reference (Figure 4.13A) or Epileptic group (Figure 4.13B) as controls. This might suggest that this panel of microRNAs could be not suitable for discriminating gliomas depending on the grade of malignancy and this result could be probably due to the choice of microRNAs based on their role specifically in GBM (grade IV).

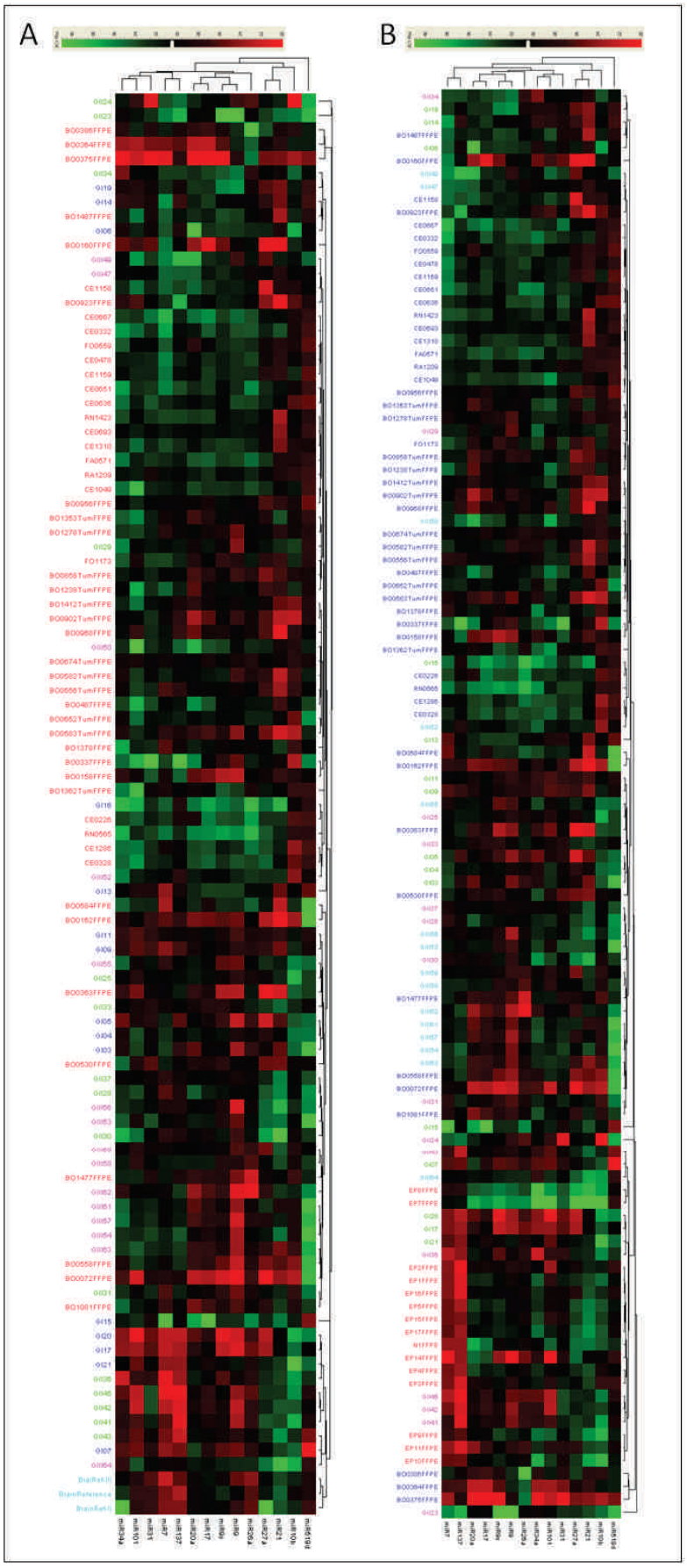


Figure 4.13. Unsupervised hierarchical clustering analysis of 4 tumor groups for the 14 microRNAs analyzed.

A) Clustering analysis of the 50 GBMs (in red), 15 Grade I (blue), 15 Grade II (green) and 15 Grade III (purple), all normalized on Ambion Commercial reference run 3 times (indicated in light blue).

B) Clustering analysis of the 50 GBMs (in blue), 15 Grade I (green), 15 Grade II (purple) and 15 Grade III (light blue), all normalized on Epileptic group (15 cases indicated in red).

GI: Grade I; GII: Grade II; GIII: Grade III; EP: Epileptic samples; BO-: anonymous code for GBM samples.

4.6.4 Differences in microRNAs expression analysis among grade I, II, III e IV gliomas

Spearman correlation between the 4 tumor groups indicated, that there was the best correlation between high grade gliomas, grade IV (GBM) and III (Table 4.15) both considering Ambion Commercial Reference and Epileptic tissues as controls.

A	vs Ref	Grade IV	Grade I	Grade II	Grade III
	Grade IV	/	0,4592	0,3508	**0,6215**
	Grade I	0,4592	/	0,3569	0,2848
	Grade II	0,3508	0,3569	/	0,4320
	Grade III	**0,6215**	0,2848	0,4320	/

B	vs EP	Grade IV	Grade I	Grade II	Grade III
	Grade IV	/	0,4702	0,3138	**0,5872**
	Grade I	0,4702	/	0,3186	0,2538
	Grade II	0,3138	0,3186	/	0,4175
	Grade III	**0,5872**	0,2538	0,4175	/

Table 4.15. Spearman correlation values between microRNAs profiles obtained for the 4 tumor groups ($p<0.0001$)**.** A) Spearman correlation performed on profiles obtained using Ambion Commercial Reference as control. The best correlation value was obtained between grade III and IV gliomas (in bold). B) Spearman correlation performed on profiles obtained using Epileptic Group as control. The best correlation value was obtained between grade III and IV gliomas (in bold). Ref: Ambion commercial reference; Ep: epileptic group.

A Mann-Whitney test was further performed, comparing microRNAs expression levels of GBM profiles with grade I, II or III gliomas profiles. (Figures 4.14 A and B - see Appendix E for higher magnification). As showed in Figures 4.14 A and B, we considered microRNAs profiles obtained using both Ambion Commercial Reference (Figure 4.14A) and Epileptic Group (Figure 4.14B) as controls. This analysis let us to understand which microRNAs were statistically different among glioma groups, focusing our attention not only on those microRNAs which have showed different regulation status among tumor groups but also on those which

shared the same deregulation status to investigate possible statistically significant differences in expression levels.

These results obtained performing Mann-Whitney test are showed in Figures 4.14 A and B and summarized in Table 4.16.

Two microRNAs (miR-7 and miR-137) were downregulated in all glioma groups even if with some significant differences in expression levels as, for example, between GBM and grade II for miR-7 or between GBM and grade I for miR-137.

Using the Commercial Ambion reference, also microRNA-26a was downregulated in GBM as well as in all other tumor groups (Tables 4.7 and 4.14 and Figure 4.14A) with statistically different levels of expression: in grade I ($p<0.5$), grade II ($p<0.001$) and grade III ($p<0.5$) (Table 4.16 and Figures 4.14A).

Considering the profiles obtained using the epileptic reference, miR-26a resulted downregulated only in grade IV brain tumors, even if the same significant differences with the other 3 groups were manteined (Figure 4.14B).

The same consideration could be proposed for miR-31 which resulted downregulated in all tumor groups, using Ambion reference as control (Tables 4.7 and 4.14 and Figure 4.14A). In this case we obtained a statistical different level of expression between GBM and grade I glioma samples (Table 4.16 and Figures 4.14A). On the contrary, using Epileptic tissues as non-neoplastic control group miRNA-31 was not-deregulated in grade I, grade II and grade III brain neoplasia (Table 4.16 and Figures 4.14B).

Moreover other 3 microRNAs shared the same deregulation status in high grade gliomas (grade III and IV): miR-34a was upregulated in low grade gliomas while is not deregulated in high grade tumors (Table 4.16 and Figures 4.14 A and B); miR-101 was downregulated only in high grade gliomas (Table 4.16 and Figures 4.14 A and B) and finally miR-10b was upregulated in high grade gliomas (grade III and IV) while was significantly downregulated in low grade gliomas (grade I and II) (Table 4.16). In particular the last microRNA resulted downregulated in grade I (Table 4.16 and Figures 4.14 A and B), while in grade II it was downregulated (Table 4.16 and Figure 4.14A) or not deregulated (Table 4.16 and Figure 4.14B), depending on the non-neoplastic reference (Ambion or Epileptic respectively).

Among the remnant 4 microRNAs, miR-9 was downregulated in GBM, but it is not deregulated in grade I, grade II and grade III brain neoplasia (Table 4.16 and Figures 4.14 A and B).

MicroRNA-21, upregulated in GBM, showed high level of expression even in grade I, but it was not deregulated in grade II (Table 4.16 and Figures 4.14 A and B). In grade III miRNA-21 showed overexpression only when compared with Epileptic reference (Table 4.16 and Figure 4.14B).

MicroRNA-27a was upregulated in GBM and in grade I tumors with a not statistical different level of expression (Table 4.16 and Figures 4.14 A and B). On the contrary it resulted not deregulated in grade II and grade III brain tumors (Table 4.16 and Figures 4.14 A and B).

Finally miR-519d was not deregulated in GBM and in grade I brain neoplasia (Table 4.16 and Figures 4.14 A and B). On the contrary it resulted downregulated in grade II and grade III brain tumors (Table 4.16 and Figures 4.14 A and B).

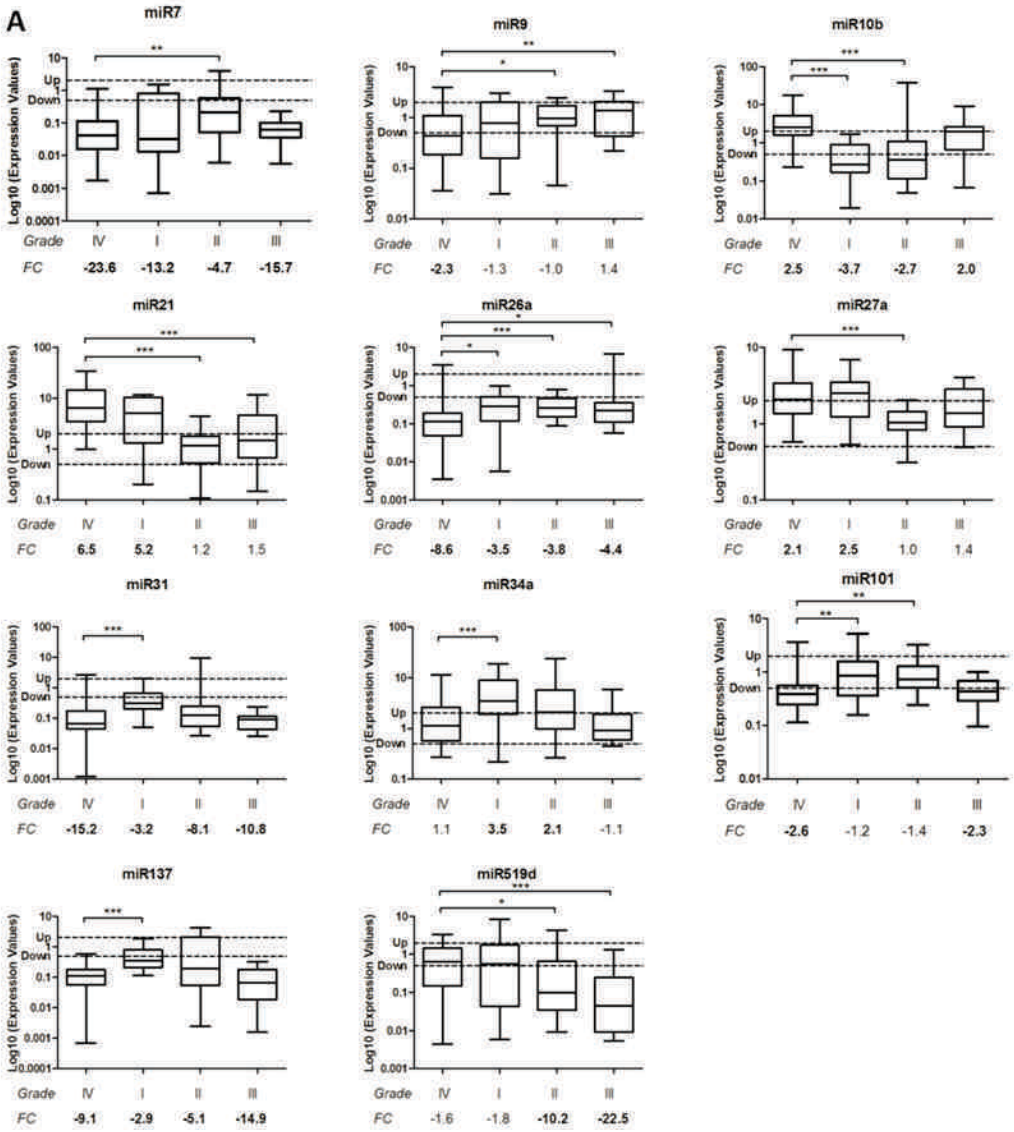


Figure 4.14A. Differences in miRNAs expression among the 4 tumor groups. The analysis was performed using Ambion Commercial Reference as control. Box plots show microRNAs significantly different between GBM Group (Grade IV) and each one of the other 3 tumor groups. In Y-axis is indicated the microRNA expression level: "Up" and "Down" lines highlight the cut-off of 2 fold change to consider a microRNA as deregulated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ according to Mann-Whitney test. FC: Fold Change.

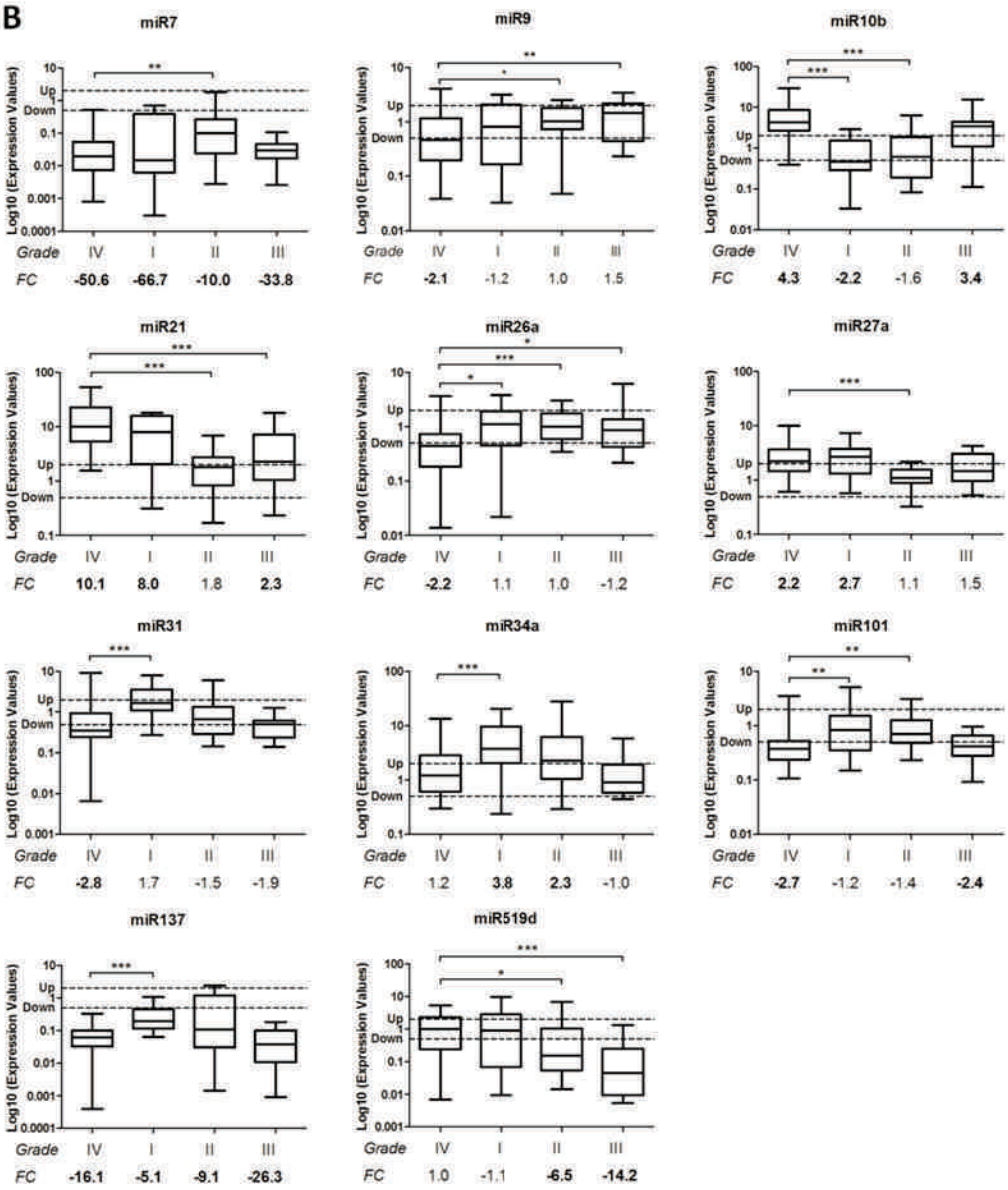


Figure 4.14B. Differences in miRNAs expression among the 4 tumor groups. The analysis was performed using Epileptic Group as control. Box plots show microRNAs significantly different between GBM Group (Grade IV) and each one of the other 3 tumor groups. In Y-axis is indicated the microRNA expression level: "Up" and "Down" lines highlight the cut-off of 2 fold change to consider a microRNA as deregulated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ according to Mann-Whitney test. FC: Fold Change.

miRNA	Brain Tumor Grade						
	IV	I	p^a	II	P^a	III	P^a
miR7	DOWN	DOWN	Ns	DOWN	**	DOWN	ns
miR9	DOWN	=	Ns	=	*	=	**
miR10b	UP	DOWN	***	DOWN/= [b]	***	UP	ns
miR21	UP	UP	ns	=	***	=/UP [b]	***
miR26a	DOWN	DOWN/= [b]	*	DOWN/= [b]	***	DOWN/= [b]	*
miR27a	UP	UP	ns	=	***	=	ns
miR31	DOWN	DOWN/= [b]	*	DOWN/= [b]	ns	DOWN/= [b]	ns
miR34a	=	UP	*	UP	ns	=	ns
miR101	DOWN	=	**	=	**	DOWN	ns
miR137	DOWN	DOWN	***	DOWN	ns	DOWN	ns
miR519d	=	=	ns	DOWN	*	DOWN	***

Table 4.16. MicroRNAs expression profiles. Ambion Commercial Reference/Epileptic Group were used as non-neoplastic controls. [a] p value is calculated according to Mann Whitney test versus grade IV profile. [b] Cases in which the results obtained using the 2 references were not the same are reported as results on Commercial Refrence/results on Epileptic tissues. Ref: Ambion Commercial Reference; EP: Epileptic Group; UP: upregulated; DOWN: downregulated; FC: Fold Change; =: not deregulated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Chapter 5:

Discussion

This project was conducted within the multicentric PERNO project (Progetto Emiliano-Romagnolo di Neuro-Oncologia, www.perno.it). The main aim was to collect and classify primary brain tumor samples and their relative epidemiologic data, about prevalence, incidence and prognosis. Among several sub-projects started within the PERNO one, this study proposed to identify a set of possible molecular markers which could be employed to assist prognosis and diagnosis evaluation in high grade glioma patients. In detail this work was focused on the expression analysis of a subset of microRNAs which could have a functional role in high grade gliomas.

Despite progresses in surgical techniques, radiotherapy, chemotherapy and "target therapy", the prognosis of primary grade IV glioblastoma, the most frequent and malignant brain tumor of the adult, remains poor [4, 8, 9]. In the last years, several biomarkers had been proposed as potentially useful parameters for prognosis, diagnosis or target therapy strategies. Among them, microRNAs could represent important molecular markers, because they play a role in important cellular processes involved in the tumorigenesis and progression of cancer and also because their expression patterns are tissue specific. Several studies have been performed in attempt to identify a specific microRNAs expression pattern of GBM [57-65] and a small subset of consistently deregulated miRNAs were functionally characterized for their activities and downstream targets involved in this tumor [73, 77-81].

For this study 19 microRNAs (miR-7, miR-9, miR-9*, miR10a, miR10b, miR-17, miR-20a, miR-21, miR-26a, miR-27a, miR-31, miR-34a, miR-101, miR-137, miR-182, miR-221, miR-222, miR-330, miR-519d) were selected for their putative role in GBM pathogenesis, according to previously published data [58-60, 62, 71, 74, 77-81, 91, 94-96, 99, 100, 113, 114].

The features that could distinguish a microRNAs profiling study from another one are mainly three: the technique performed for microRNAs expression analysis, the type of starting material and the choice of control samples enrolled in the analysis. As shown by previous published studies, microarray analysis [57, 58, 62, 73] and real time-qPCR [59, 60] remain the most commonly used techniques adopted for miRNAs investigation.

Starting from the assumption that this study was focused on the investigation of this small subset of selected microRNAs, previously published in GBM profiling studies, we decided to set up a real-time qPCR analysis to investigate the expression of these microRNAs in our cohort of GBMs. Between the two different methods for reverse transcription for real-time qPCR [112], we adopted an universal reverse transcription which included a first step where all miRNAs were elongated with a poly(A) tail and then reverse transcribed using a universal primer. A good specificity and sensitivity was ensured by using specific forward primers designed to correspond to the mature sequence of each microRNA. LNA substitutions were added in each primer to improve mismatch discriminations. In microRNAs different just for a single nucleotide (e.g. miR-10a and miR-10b), a LNA substitution was introduced in correspondence of the discriminating base.

As regard starting material, Fresh/Frozen tissues [58-60, 63, 73], formalin-fixed paraffin embedded (FFPE) specimens [64, 72] or glioblastoma cell lines [65, 77, 79, 81] are commonly chosen for GBM miRNAs profiling studies. Considering the pros and cons of different starting materials (discussed in Introduction section), the first part of this project was focused on the evaluation of the most suitable specimen for the analysis. For this reason, we have compared microRNAs expression between paired Fresh/Frozen and FFPE GBM samples.

In our study, we collected 30 paired GBMs, available both as Fresh/Frozen and FFPE specimens. MicroRNAs expression was analyzed in all 60 samples using qRT-PCR. Neoplastic cell percentage was evaluated in Fresh/Frozen tissues on a snap frozen section. MicroRNAs extraction was performed starting from 50–80 mg of not morphologically checked tissue. MicroRNAs from FFPE specimens were extracted starting from four 20 μm-thick sections according to the selected area signed on H&E control slide (FFPE-dissected), ensuring whenever possible an enrichment in neoplastic cells >90%, avoiding “normal” cells contaminations. The results of comparison between miRNAs profiles of the two groups showed a good correlation ($r = 0.7916$): only 3 microRNAs (miR-137, miR-20a and miR-21) were differentially expressed in Fresh/Frozen samples if compared with FFPE-dissected ones, while the remnant microRNAs did not shown any significant statistically differences in expression values. Moreover considering

each specimens pair, good correlation values ($r > 0.65$) were obtained between the microRNAs expression levels in 25 out of the 30 sample pairs analyzed.

The low correlation in the remnant 5 cases could be due to enrichment in neoplastic cells in dissected samples in comparison to Fresh/Frozen ones. Analyzing the same cases without performing dissection, we obtained in 3 out of 4 available cases a better correlation value comparing Fresh/Frozen and FFPE-not dissected samples. These results suggest that the previous discrepant results could be ascribed to the presence of non-neoplastic cells in Fresh/Frozen tissues.

To the best of our knowledge, this is the first analysis in glioblastoma samples that compares microRNAs expression between paired FFPE-dissected samples and Fresh/Frozen specimens. As previously published for other tissues, these results demonstrate that miRNAs expression analysis is feasible and results are comparable starting from FFPE-dissected GBMs as well as from Fresh/Frozen ones [120]. Some significant differences in microRNAs expression levels had been showed suggesting that the possible presence, in unknown amount, of non-neoplastic cells in Fresh/Frozen tissues (endothelial, lymphocytes, normal glial cells) could influence microRNAs expression results [120].

Another key point in microRNAs expression analysis is the choice of non-neoplastic samples to use as reference controls. The difficulty to retrieve normal tissues in brain samples let to choose several non-neoplastic resources and the most commonly used references samples are normal adjacent the tumor specimens [58, 73, 121], normal brain tissues obtained from epileptic patient surgeries [37, 59, 60, 63], cell lines [65, 77] and commercial pools of RNAs obtained from normal brain areas [67, 71]. To investigate if microRNAs profiles could be different depending on the non-neoplastic control used, we compared GBM microRNAs expression results obtained using three different non-neoplastic references, recreating three frequent experimental conditions. We collected normal adjacent the tumor specimens (N-Ad), all from the GBM samples group, normal brain tissues retrieved from epileptic patient surgeries (Ep) and a commercial pool of brain RNAs (FirstChoiceH Human Brain Reference RNA from Ambion; Ref).

Analyzing these 3 non-neoplastic references for the same panel of 19 microRNAs, a good correlation value ($r > 0.65$) was obtained comparing the three profiles and the best correlation was observed between Ambion Commercial Reference and Epileptic Group profiles ($r = 0.848$). Considering the normal adjacent the tumor, a lower correlation value was obtained, both in comparison with epileptic tissues ($r = 0.702$) and commercial reference ($r = 0.724$). This result might be explained by possible alterations in normal tissues adjacent the tumor, due to influence of surrounding neoplastic cells. Analyzing the median expression values of each microRNA in the three references, 9 microRNAs (miR-7, miR-9, miR-10a, miR-10b, miR-26a, miR-27a, miR-31, miR-137, and miR-182) resulted statistically different comparing the three groups together (Kruskal Wallis test, $p<0.05$). Furthermore, considering groups in pairs (Mann-Whitney test, $p<0.05$), the significant differences obtained with Kruskal Wallis test were confirmed and in addition statistical significant differences even in microRNA-101 (between N-Ad and Ref) and microRNA-519d (between N-Ad and EP) were observed [122].

These discrepancies represented an interesting result because confirmed that, although there was a good correlation among microRNAs expression profiles, several differences in microRNAs expression distinguish a non-neoplastic control from another one. These differences among non-neoplastic references could be imputable to: 1) the physiological differences in mean age showed by the three control groups (for example epilepsy has an earlier onset in comparison to GBM); 2) a technical fault encountered during the analysis due to the fact that epileptic and normal adjacent the tumor samples were analyzed individually and microRNAs expression data were unified in statistical analysis (biological variability) while the commercial reference was purchased as a pool of RNAs and its analysis was repeated three times (technical variability); 3) non-neoplastic samples could have real different microRNAs expression levels [122].

Our data confirmed that the choice of the reference control in GBM microRNAs profiling study represents a crucial starting point. Moreover it is very important to compare microRNAs expression results with data obtained using similar experimental conditions (e.g. the source of tumor tissues or the reference control adopted) [120, 122].

The third part of the project was focused on the definition of a microRNAs profile of GBM group. Fifty GBM samples were collected within the PERNO project cohort and they were further analyzed for microRNAs expression.

Considering the previously obtained data, the analysis was performed on FFPE-dissected samples, to ensure a good enrichment in neoplastic cells, and GBM microRNAs profiles were obtained using all the 3 non-neoplastic references, to verify if different controls could provide different GBM microRNAs profiles.

The median expression values of the 19 microRNAs in GBM group were different according to non-neoplastic reference adopted as control. Moreover, even in those miRNAs that showed the same status, differences in fold change values can be observed (e.g. miR-7, miR-137).

The best correlation value was observed between profiles obtained using Ambion commercial reference and Epileptic group as controls. In addition these two profiles shared the higher number of microRNAs with the same regulation status (14 out of the 19 microRNAs analyzed).

To define a GBM microRNAs profile, only microRNAs deregulated in at least 2 out of the 3 profiles obtained (considering a cut-off of ±2 fold-change), were considered. Mir-10b, miR-21 and miR-27a resulted upregulated while miR-7, miR-9, miR-26a, miR-31, miR-101, miR-137, miR-222 and miR-330 resulted downregulated in GBM group. Only 4 miRNAs (miR-21, miR-7, miR-31 and miR-137) showed the same deregulation status in GBM, independently by the non-neoplastic reference. The others microRNAs not considered for GBM profile were excluded because they resulted not deregulated (miR-9*, miR-17, miR-20a, miR34a) or they showed a dysregulated (miR-10a, miR-182, miR-221 and miR-519d) status in only 1 profile out of the 3 analyzed.

Bearing in mind the small subset of selected microRNAs, we have defined a putative GBM microRNAs profile. Moreover differences observed in microRNAs expression among the 3 references described above, let to different GBMs microRNAs profiles.

Our microRNAs GBM profile resulted in accordance with profiling data previously reported in literature, except for 3 microRNAs (miR-9, miR-26a and miR-222).

Mir-9 resulted downregulated in our study (in detail resulted downregulated in GBMs versus commercial reference and versus epileptic tissues) while was reported upregulated in GBM by Ciafrè *et al.* [58] and Malzkorn *et al.* [71]: in the first study high levels of miR- 9-2 were evaluated through microarray in 5 out of 9 fresh tumors compared to their corresponding normal adjacent tissues; while in the second study the upregulation of miR-9 was determined by stem-loop realtime RT–PCR in 4 fresh secondary GBMs in comparison with their corresponding primary grade II gliomas. It should be considered that Ciafrè *et al.* [58] analyzed fresh tumor tissues, not FFPE-dissected samples, and that Malzkorn *et al.* results [71] were obtained in secondary GBMs in comparison not with non-neoplastic specimens but with primary grade II gliomas.

Our results showed a downregulation of miR-26a in GBM, both versus commercial reference and epileptic tissues, while the same miRNA was previously reported upregulated by Godlewski *et al.* [62] and Huse *et al.* [78]. Both studies used microarray technique to evaluate microRNAs expression, the first one in GBM mouse models after implantation of primary human glioma cells derived from freshly resected specimens [62], while the second study performed the analysis on 3 fresh GBM samples versus 2 normal brain specimens [78]. Also in these cases, the studies design presents significant differences if compared with our, for example the use of animal model or the analysis on fresh GBM samples.

Finally miR-222 resulted significantly downregulated in our study both in comparison with epileptic and normal adjacent the tumor tissues. Although miR-221 and miR-222 are encoded in the same genomic cluster on the X chromosome (Entrez Gene www.ncbi.nlm.gov), miR-221 resulted downregulated only versus epileptic tissues. Conti *et al.* [60], Ciafrè *et al.* [58], Quintavalle *et al.* [97] and Gillies *et al.* [93] reported an upregulation of miR221/222 cluster in GBM: Quintavalle *et al.* study [97] assessed the specific upregulation of miR-222 using microarray technique in GBM cell lines in comparison with non-tumorigenic T98G cells; the others evaluated only miR-221 levels, considering that miR-222, could shared the same regulation [58, 60, 93]. Conti *et al.* [60] and Gillies *et al.* [93] used real-time PCR technique on fresh GBM tissues in comparison with epileptic specimens and on cell lines, respectively; while Ciafrè *et al.* [58] used

microarray on fresh GBM and normal adjacent tissues as explained before. In another study instead Slaby *et al.*[64] found, by stem-loop RT-PCR, miR-221 and miR-222 downregulated in FFPE-dissected GBMs (ensuring >90% of tumor cells) in comparison to non malignant brain tissues derived from areas surrounding arteriovenous malformation (AVM). Authors explained this result as due to the choice of normal brain samples: traces of micro-capillaries from around the AVM could be present and, considering that endothelial cells generally showed high levels of miR-221/222, this could lead to discrepant results with other studies [64].

Considering that we analyzed 50 GBM FFPE-dissected samples, adopting different non-neoplastic references for the analysis and using qRT-PCR with specific LNA primers, these discrepant data could be explained by different experimental conditions in which those cited results were obtained.

Even if the functional analysis of possible mRNA targets of deregulated microRNAs was not a purpose of this project, a preliminary analysis of putative targets controlled by the selected microRNAs was performed. Considering only the 11 microRNAs (miR-7, miR-9, miR-10b, miR-21, miR-26a, miR-27a, miR-31, miR-101, miR-137, miR-222, miR-330) with a significant deregulation in GBMs, their experimentally validated targets were investigated using three online tools (miRecords, miRTarBase and miRWalk). Fifty-six validated targets, shared by all bioinformatics tools, were further analyzed for their molecular functions and pathways involvement using PANTHER database. Interleukin signaling, insulin/IGF pathway-MAPKK/MAPK cascade, gonadotropin-releasing hormone receptor (GNRHR), angiogenesis and Insulin/IGF-PKB pathways resulted significantly represented by this list of targets and previous studies have demonstrated their decisive role in glioblastoma pathogenesis [11, 14, 123-129].

The aim of the last part of this study was to compare microRNAs expression between GBM and gliomas of lower grade (grade I, II and III). We evaluated if there were some differences in microRNAs expression associated with different grade of malignancy. We focused our attention not only on those microRNAs which showed different regulation status among tumor groups but also on those

with the same deregulation status for investigating statistically significant differences in expression levels.

As reference controls, due to limited amount of normal adjacent the tumor tissues in grade I, II and III, we adopted only epileptic tissues and commercial reference. For this reason we focused the attention on 14 microRNAs that have shared the same deregulation status in GBM group profiles using these two controls. We analyzed the expression of these 14 microRNAs in glioma groups of grade I, II and III, comparing the results with the GBM profiles obtained using the same references.

An unsupervised hierarchical clustering analysis revealed that there was not a clear separation between different grade gliomas depending on microRNAs expression, independently by the reference used. This result indicates that this panel of microRNAs could be not suitable for discriminating gliomas simply on the grade of malignancy and it could be explained by the arbitrary choice of a small subset of microRNAs known to have a role specifically in grade IV gliomas. Analyzing microRNAs expression profiles of the 4 tumor groups, the best correlation value was obtained between high grade glioma profiles (grade III and GBM). Only two microRNAs (miR-7 and miR-137) showed the same deregulation status among the 4 tumor groups, independently by the non-neoplastic control used, even if with some significant differences in expression levels as, for example, miR-7 between GBM and grade II or miR-137 between GBM and grade I. MiR-26a and miR-31 resulted downregulated in GBM as well as in all other tumor groups using only Ambion reference as control. Mir-26a presented statistically different levels of expression in all grades when compared to GBM, while miR-31 expression level was significantly different only in grade I gliomas. Considering the profiles obtained using the epileptic reference, both microRNAs resulted downregulated only in grade IV brain tumors, even if the same significant differences showed in the analysis with the commercial reference were maintained.

Intriguingly 3 microRNAs shared the same deregulation status only in high grade gliomas (grade III and IV): miR-34a, stable in high grade tumors, was upregulated in low grade gliomas (grade I and II), miR-101 was downregulated only in high

grade gliomas and finally miR-10b resulted upregulated in high grade gliomas while was significantly downregulated in low grade gliomas. MiR-34a was previously reported in GBM as downregulated and previous studies have analyzed its possible role in glioblastoma pathogenesis [92, 94, 98]. Oncogenes like c-met, Notch1, Notch2, CDK6, PDGFRA and SMAD4, in TGFβ/SMAD pathway, have been validated as targets of miR-34a and they in fact resulted overexpressed in GBM [92, 94, 98]. Although miR-34a resulted not deregulated in our high grade glioma groups, it showed an upregulation in low grade tumors, suggesting a possible association between miR-34a loss and GBM pathogenesis.

MiR-101 resulted to be downregulated, in accordance with previous studies [59, 81]. In particular we obtained that this miRNA deregulation could be present only in association with high grade of malignancy. Smits *et al.* [81] have previously demonstrated one possible role of miR-101 in GBM progression: they obtained lower levels of miR-101 in comparison with grade II-III gliomas and they reported the association with overexpression of miR-101 target EZH2, which influences proliferation, invasion and angiogenesis.

Finally miR-10b resulted significantly upregulated in high grade gliomas while was downregulated in low grade tumor groups. The data about GBM are in line with results previously published by other studies [58, 59, 73, 91]. In particular the study by Gabriely *et al.* [91] demonstrated the role of miR-10b in cell proliferation and in cell cycle regulation targeting Bim (a pro-apoptotic factor) and p16/CDKN2A - p21/CDKN1B, respectively. Sasayama *et al.* [73] reported that miR-10b expression was associated with glioma grade of malignancy and its expression was significantly lower in low grade gliomas compared to high grade astrocytic tumors. Moreover they demonstrated that levels of invasive factors RhoC and urokinase-type plasminogen activator receptor (uPAR) were inversely correlated to miR-10b expression, indicating that it might play a key role in invasion features of gliomas. All these data suggested that a silencing of miR-10b could be an interesting therapeutic strategy for GBM treatment and a study of miR-10b expression in primary low grade gliomas and in corresponding secondary GBM lesions could be interesting to evaluate a correlation with tumor progression.

Chapter 6:

Conclusions

We investigated the expression of a panel of 19 microRNAs in brain tumors, focusing our attention on GBM expression signature.

The present study leads to the following considerations:

1) the feasibility to perform microRNAs profiling study starting from FFPE specimens brought three main known advantages: a good disposal of archival GBM samples, a greater probability to retrieve normal brain samples which are very difficult to obtain as fresh tissues and the possibility to verify the real percentage of tumor cells of the analyzed sample (limiting possible contaminations of non-neoplastic cells);

2) the comparison of microRNAs expression using different non-neoplastic references highlighted that a GBM microRNAs profile could be strictly dependent, not only by the source of tissue, but also by the non-neoplastic control chosen;

3) GBM microRNAs profile has showed mir-10b, miR-21 and miR-27a as upregulated, while miR-7, miR-9, miR-26a, miR-31, miR-101, miR-137, miR-222 and miR-330 resulted downregulated;

4) comparing expression results in GBM group with glioma groups of lower grade (grade I, II and III), we found 3 microRNAs with a different regulation status between high grade gliomas (grade III and IV) and low grade gliomas (I and II): miR-34a, stable in high grade tumors, was upregulated in low grade gliomas; miR-101 downregulated only in high grade gliomas and miR-10b upregulated in high grade gliomas while was significantly downregulated in low grade gliomas. Among these the last microRNA, miR-10b, could be interesting both as therapeutic strategy for GBM treatment and for its possible association with tumor progression.

We concluded this project affirming that in GBM microRNAs profiling studies is recommend to compare miRNAs expression results with data previously obtained in similar experimental conditions, considering the number of cases analyzed, the type of selected tissue, the non-neoplastic control chosen and the technique adopted for microRNAs expression analysis.

Bearing in mind these considerations, further investigations about a specific signature of microRNAs expression in GBM could be fundamental to discover

new specific therapeutic strategies, for example silencing a microRNA selectively overexpressed in GBM, and possible correlations with glioma progression (for example extending the analysis on low grade primary glioma tumors and on their corresponding secondary high grade lesions).

Appendix

Appendix A

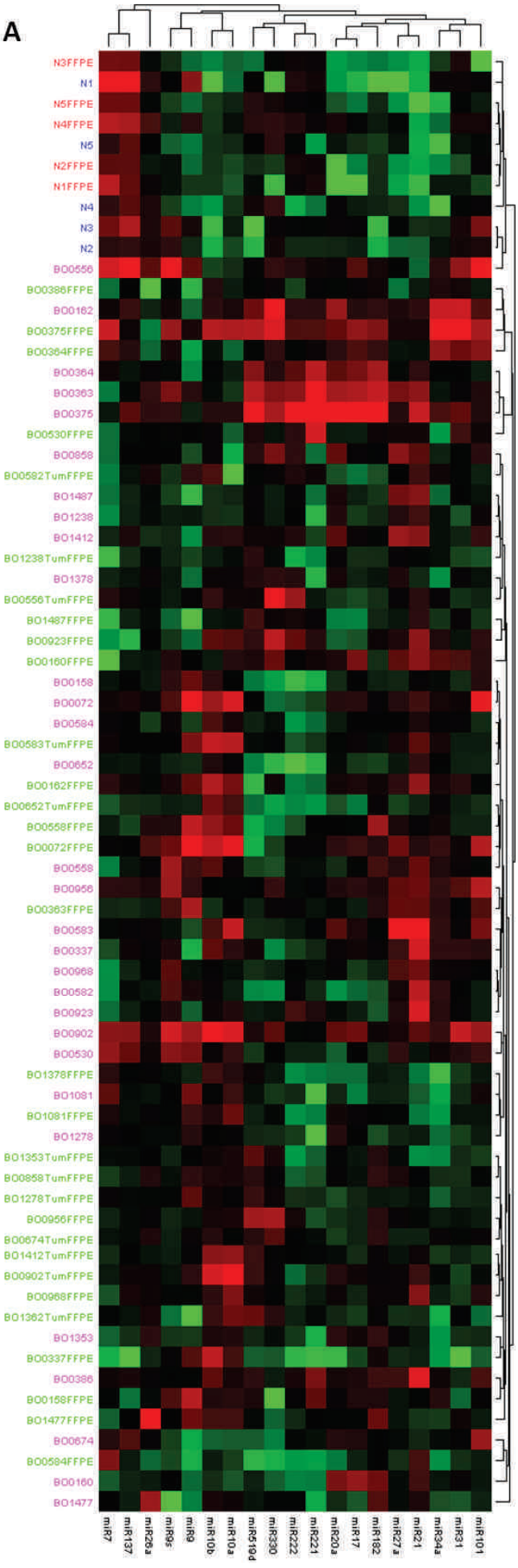
Higher magnification of Figure 4.2. Unsupervised hierarchical clustering analysis of 30 Fresh/Frozen and FFPE-dissected GBM for the 19 microRNAs analyzed.

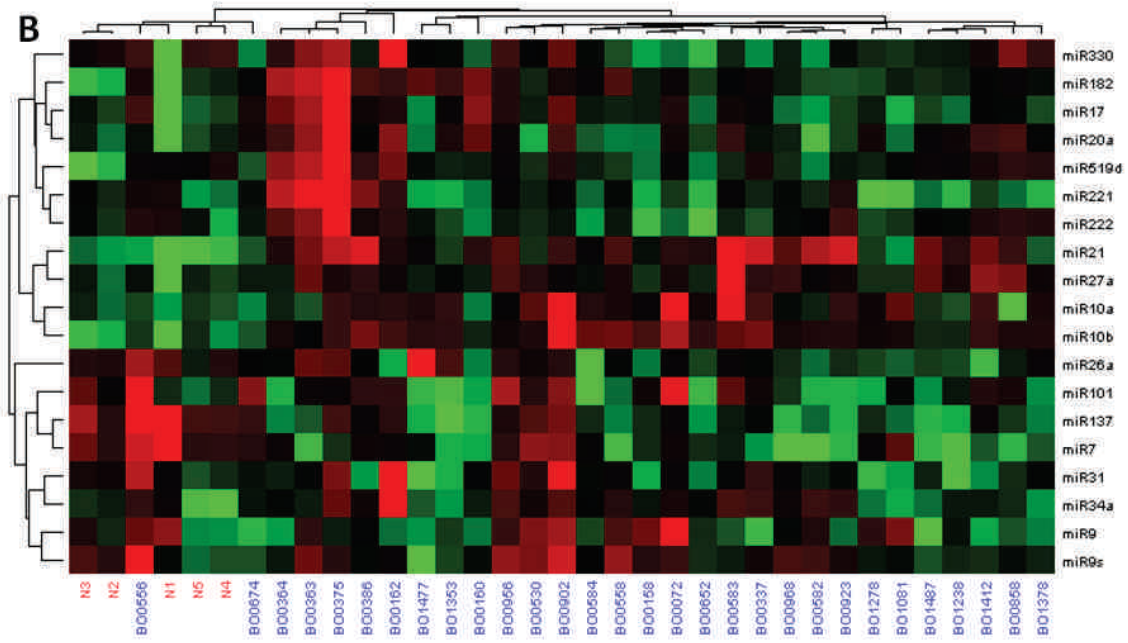
A) Unsupervised clustering analysis of the 30 paired samples, both tumors and normal brain specimens. FFPE-dissected GBM samples are written in green, Fresh/Frozen GBM in pink, Fresh/Frozen normal brain tissues in blue and FFPE control samples in red.

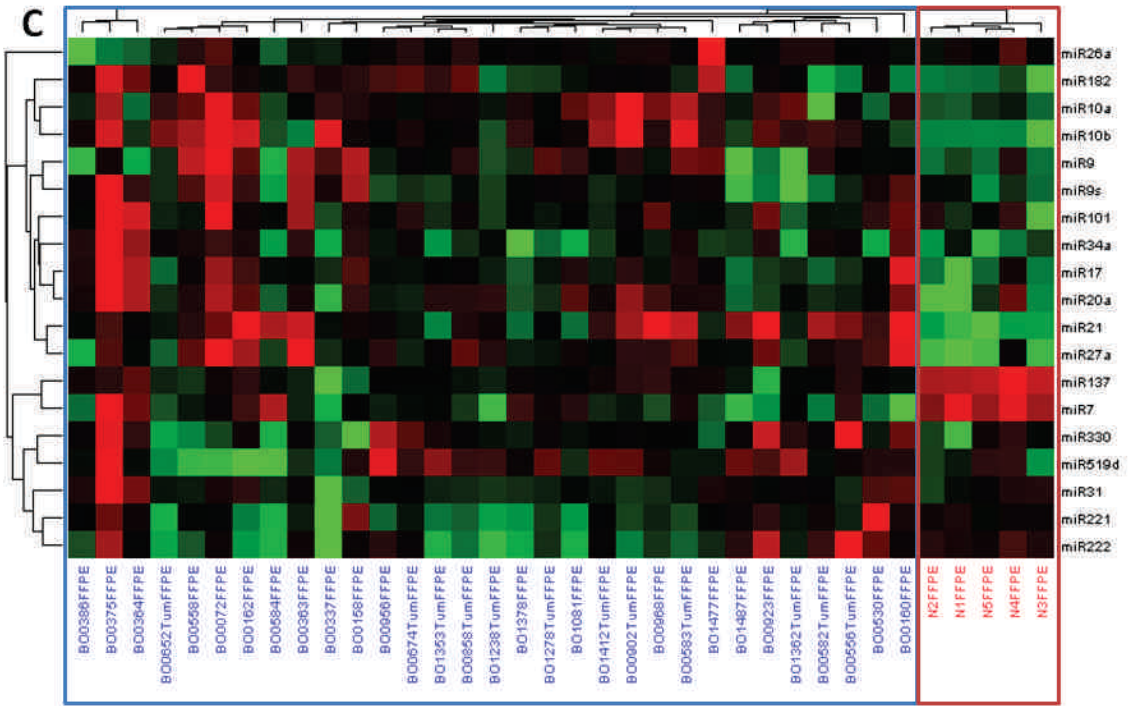
B) Unsupervised clustering analysis of the 30 Fresh/Frozen GBM samples: GBM group is written in blue and normal brain samples in red.

C) Unsupervised clustering analysis of the 30 FFPE GBM samples: GBM group is represented in blue and normal brain samples in red. Blue square highlights GBM group and red square the control group.

N: normal sample; BO-: anonymous code for GBM samples.


A
N3FFPE
N1
N5FFPE
N4FFPE
N5
N2FFPE
N1FFPE
N4
N3
N2
BO0556
BO0386FFPE
BO0162
BO0375FFPE
BO0364FFPE
BO0364
BO0363
BO0375
BO0530FFPE
BO0858
BO0592TumFFPE
BO1487
BO1238
BO1412
BO1238TumFFPE
BO1378
BO0556TumFFPE
BO1487FFPE
BO0923FFPE
BO0160FFPE
BO0158
BO0072
BO0584
BO0583TumFFPE
BO0652
BO0162FFPE
BO0652TumFFPE
BO0558FFPE
BO0072FFPE
BO0558
BO0956
BO0363FFPE
BO0583
BO0337
BO0968
BO0582
BO0923
BO0902
BO0530
BO1378FFPE
BO1081
BO1081FFPE
BO1278
BO1353TumFFPE
BO0858TumFFPE
BO1278TumFFPE
BO0956FFPE
BO0674TumFFPE
BO1412TumFFPE
BO0902TumFFPE
BO0968FFPE
BO1362TumFFPE
BO1353
BO0337FFPE
BO0386
BO0158FFPE
BO1477FFPE
BO0674
BO0584FFPE
BO0160
BO1477
miR7
miR137
miR26a
miR9s
miR9
miR10b
miR10a
miR519d
miR330
miR222
miR221
miR20a
miR17
miR182
miR27a
miR21
miR34a
miR31
miR101

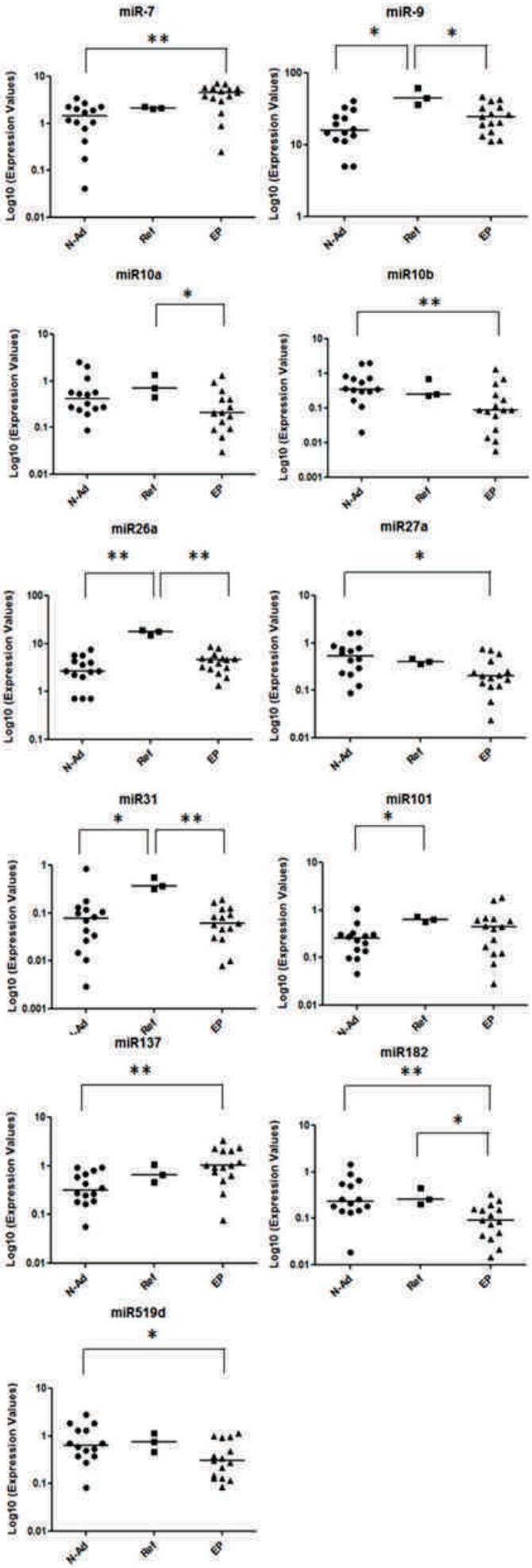

B
miR330
miR182
miR17
miR20a
miR519d
miR221
miR222
miR21
miR27a
miR10a
miR10b
miR26a
miR101
miR137
miR7
miR31
miR34a
miR9
miR9s
N3
N2
BO0566
N1
N5
N4
BO0674
BO0364
BO0363
BO0375
BO0386
BO0162
BO1477
BO1353
BO0160
BO0956
BO0530
BO0902
BO0584
BO0558
BO0158
BO0072
BO0652
BO0583
BO0337
BO0968
BO0582
BO0923
BO1278
BO1081
BO1487
BO1238
BO1412
BO0858
BO1378


C
miR26a
miR182
miR10a
miR10b
miR9
miR9s
miR101
miR34a
miR17
miR20a
miR21
miR27a
miR137
miR7
miR330
miR519d
miR31
miR221
miR222
BO0386FFPE
BO0375FFPE
BO0364FFPE
BO0652TumFFPE
BO0558FFPE
BO0072FFPE
BO0162FFPE
BO0584FFPE
BO0363FFPE
BO0337FFPE
BO0158FFPE
BO0956FFPE
BO0674TumFFPE
BO1353TumFFPE
BO0858TumFFPE
BO1238TumFFPE
BO1378FFPE
BO1278TumFFPE
BO1081FFPE
BO1412TumFFPE
BO0902TumFFPE
BO0968FFPE
BO0583TumFFPE
BO1477FFPE
BO1487FFPE
BO0923FFPE
BO1362TumFFPE
BO0582TumFFPE
BO0556TumFFPE
BO0530FFPE
BO0160FFPE
N2FFPE
N1FFPE
N5FFPE
N4FFPE
N3FFPE

Appendix B

Higher magnification of Figure 4.6. Differences in microRNAs expression among three non-neoplastic references.

Scatter plots show microRNAs significantly different among the three groups. * $p<0.05$, ** $p<0.01$ according to Mann-Whitney test. N-Ad: Normal adjacent the tumor; Ref: Ambion Commercial reference; EP: epileptic tissue.


miR-7
**
Log10 (Expression Values)
10
1
0.1
0.01
N-Ad
Rel
EP
miR-9
*
*
Log10 (Expression Values)
100
10
1
N-Ad
Rel
EP
miR10a
*
Log10 (Expression Values)
10
1
0.1
0.01
N-Ad
Rel
EP
miR10b
**
Log10 (Expression Values)
10
1
0.1
0.01
0.001
N-Ad
Rel
EP
miR26a
**
**
Log10 (Expression Values)
100
10
1
0.1
N-Ad
Rel
EP
miR27a
*
Log10 (Expression Values)
10
1
0.1
0.01
N-Ad
Rel
EP
miR31
*
**
Log10 (Expression Values)
1
0.1
0.01
0.001
Rel
EP
miR101
*
Log10 (Expression Values)
10
1
0.1
0.01
Rel
EP
miR137
**
Log10 (Expression Values)
10
1
0.1
0.01
N-Ad
Rel
EP
miR182
**
*
Log10 (Expression Values)
10
1
0.1
0.01
N-Ad
Rel
EP
miR519d
*
Log10 (Expression Values)
10
1
0.1
0.01
N-Ad
Rel
EP

Appendix C

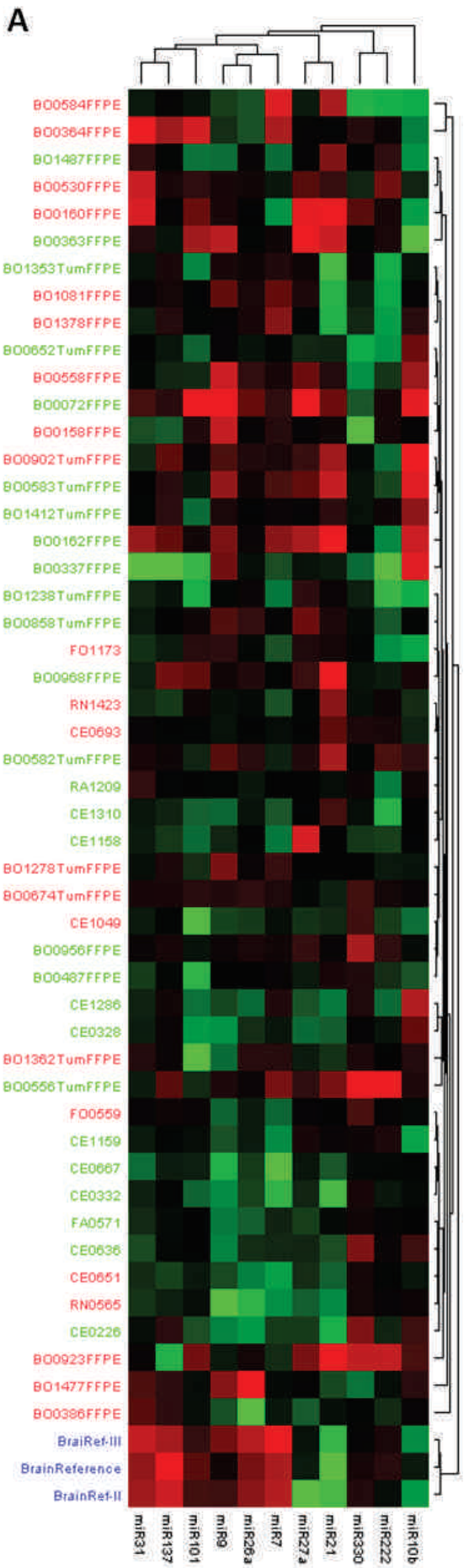
Higher magnification of Figure 4.8. Unsupervised hierarchical clustering analysis of MET-GBM and UMET-GBM for the 11 selected microRNAs.

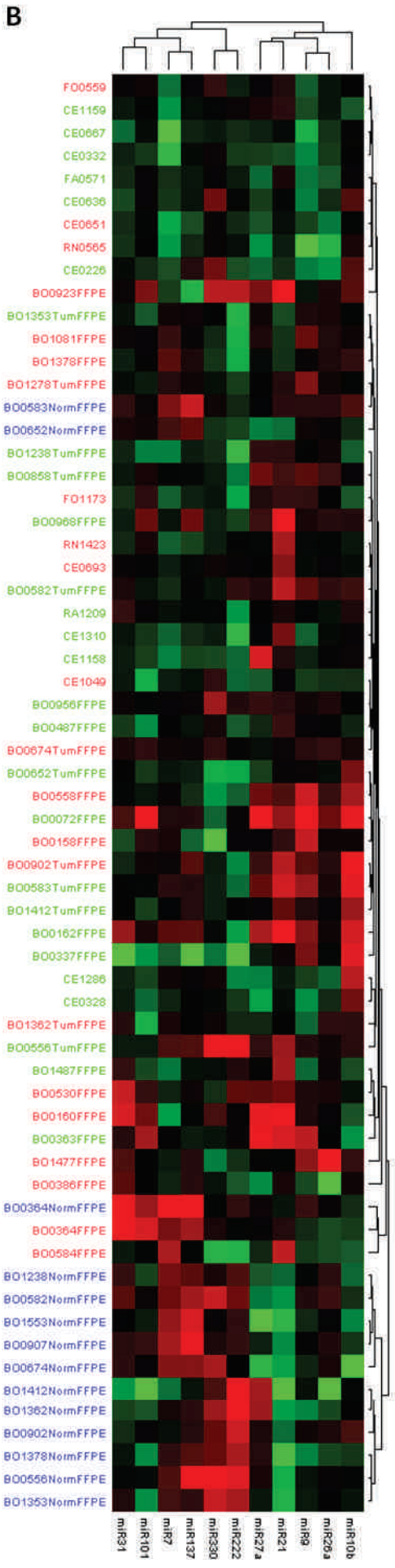
A) Unsupervised clustering analysis of MET-GBM and UM-GBM samples was performed using, Ambion Commercial Reference as control.

B) Unsupervised clustering analysis of MET-GBM and UM-GBM samples was performed using normal adjacent the tumor group as control.

C) Unsupervised clustering analysis of MET-GBM and UM-GBM samples was performed using epileptic control group.

MET-GBM samples are written in red, UMET-GBM in green, non-neoplastic control samples in blue.


A
BO0584FFPE
BO0364FFPE
BO1487FFPE
BO0530FFPE
BO0160FFPE
BO0363FFPE
BO1353TumFFPE
BO1081FFPE
BO1378FFPE
BO0652TumFFPE
BO0558FFPE
BO0072FFPE
BO0158FFPE
BO0902TumFFPE
BO0583TumFFPE
BO1412TumFFPE
BO0162FFPE
BO0337FFPE
BO1238TumFFPE
BO0858TumFFPE
FO1173
BO0968FFPE
RN1423
CE0693
BO0582TumFFPE
RA1209
CE1310
CE1158
BO1278TumFFPE
BO0674TumFFPE
CE1049
BO0956FFPE
BO0487FFPE
CE1286
CE0328
BO1362TumFFPE
BO0556TumFFPE
FO0559
CE1159
CE0667
CE0332
FA0571
CE0636
CE0651
RN0565
CE0226
BO0923FFPE
BO1477FFPE
BO0396FFPE
BraiRef-III
BrainReference
BrainRef-II
miR31
miR137
miR101
miR9
miR26a
miR7
miR27a
miR21
miR330
miR222
miR10b


B
FO0559
CE1159
CE0667
CE0332
FA0571
CE0636
CE0651
RN0565
CE0226
BO0923FFPE
BO1353TumFFPE
BO1081FFPE
BO1378FFPE
BO1278TumFFPE
BO0583NormFFPE
BO0652NormFFPE
BO1238TumFFPE
BO0858TumFFPE
FO1173
BO0968FFPE
RN1423
CE0693
BO0582TumFFPE
RA1209
CE1310
CE1158
CE1049
BO0956FFPE
BO0487FFPE
BO0674TumFFPE
BO0652TumFFPE
BO0558FFPE
BO0072FFPE
BO0158FFPE
BO0902TumFFPE
BO0583TumFFPE
BO1412TumFFPE
BO0162FFPE
BO0337FFPE
CE1286
CE0328
BO1362TumFFPE
BO0556TumFFPE
BO1487FFPE
BO0530FFPE
BO0160FFPE
BO0363FFPE
BO1477FFPE
BO0386FFPE
BO0364NormFFPE
BO0364FFPE
BO0584FFPE
BO1238NormFFPE
BO0592NormFFPE
BO1553NormFFPE
BO0907NormFFPE
BO0674NormFFPE
BO1412NormFFPE
BO1362NormFFPE
BO0902NormFFPE
BO1378NormFFPE
BO0556NormFFPE
BO1353NormFFPE
miR31
miR101
miR7
miR137
miR330
miR222
miR27a
miR21
miR9
miR26a
miR10b


C
BO0584FFPE
BO0364FFPE
BO1487FFPE
BO0530FFPE
BO0160FFPE
BO0363FFPE
BO1353TumFFPE
BO1081FFPE
BO1378FFPE
BO0652TumFFPE
BO0558FFPE
BO0072FFPE
BO0158FFPE
BO0902TumFFPE
BO0583TumFFPE
BO1412TumFFPE
BO0162FFPE
BO0337FFPE
BO1238TumFFPE
BO0858TumFFPE
FO1173
BO0968FFPE
RN1423
CE0693
BO0582TumFFPE
RA1209
CE1310
CE1158
BO1278TumFFPE
BO0674TumFFPE
CE1049
BO0956FFPE
BO0487FFPE
CE1286
CE0328
BO1362TumFFPE
BO0556TumFFPE
FO0559
CE1159
CE0667
CE0332
FA0571
CE0636
CE0651
RN0565
CE0226
BO0923FFPE
BO1477FFPE
BO0386FFPE
EP11FFPE
EP10FFPE
EP5FFPE
EP17FFPE
EP15FFPE
EP16FFPE
EP14FFPE
N1FFPE
EP1FFPE
EP4FFPE
EP3FFPE
EP2FFPE
EP9FFPE
EP7FFPE
EP8FFPE
miR27a
miR21
miR10b
miR222
miR101
miR31
miR7
miR137
miR9
miR26a
miR330

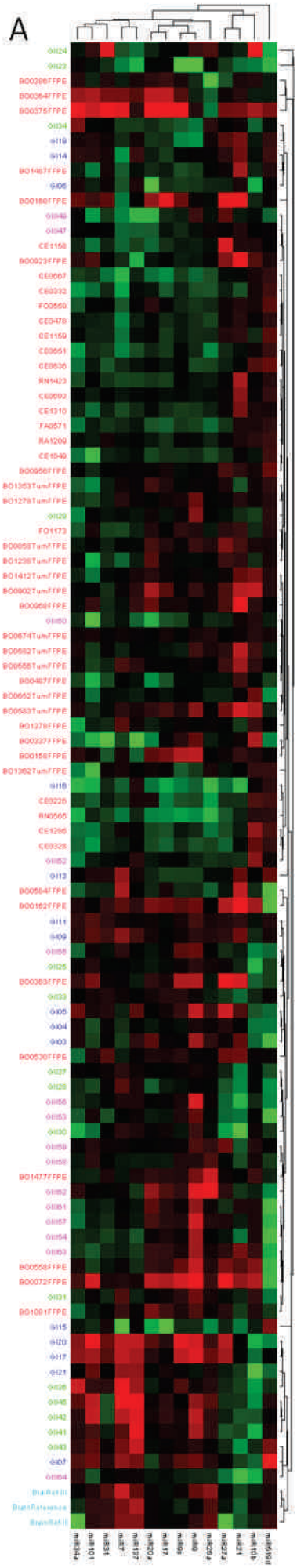
Appendix D

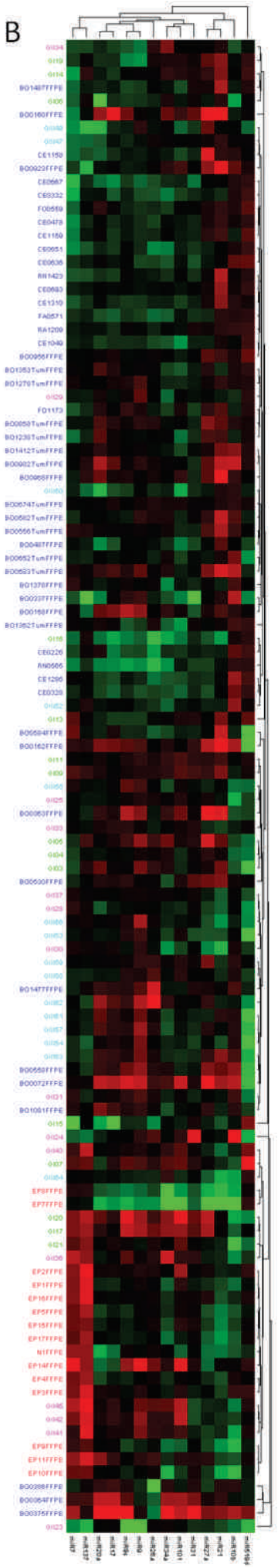
Higher magnification of Figure 4.13: Unsupervised hierarchical clustering analysis of 4 tumor groups for the 14 microRNAs analyzed.

A) Unsupervised clustering analysis of the 50 GBMs (in red), 15 Grade I (blue), 15 Grade II (green) and 15 Grade III (purple), all normalized on Ambion Commercial reference run 3 times (indicated in light blue).

B) Unsupervised clustering analysis of the 50 GBMs (in blue), 15 Grade I (green), 15 Grade II (purple) and 15 Grade III (light blue), all normalized on Epileptic group (15 cases indicated in red).

GI: Grade I; GII: Grade II; GIII: Grade III; EP: Epileptic samples; BO-: anonymous code for GBM samples.

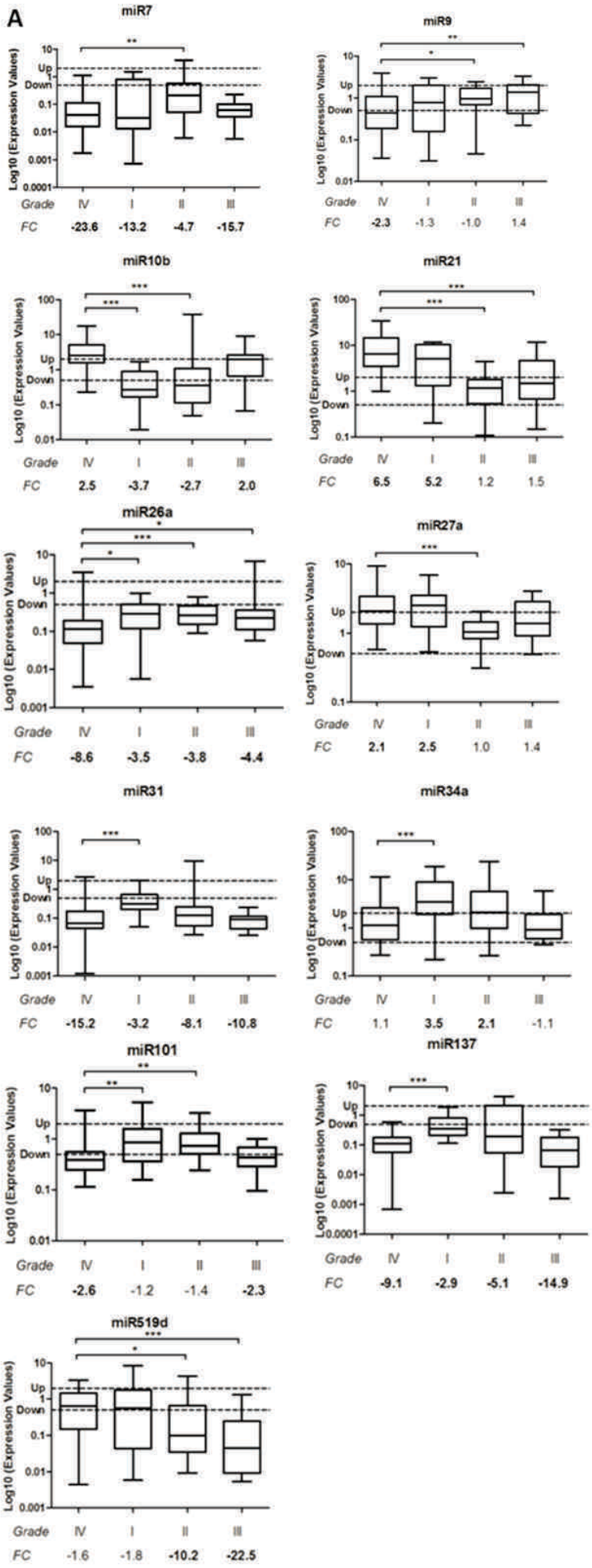

A

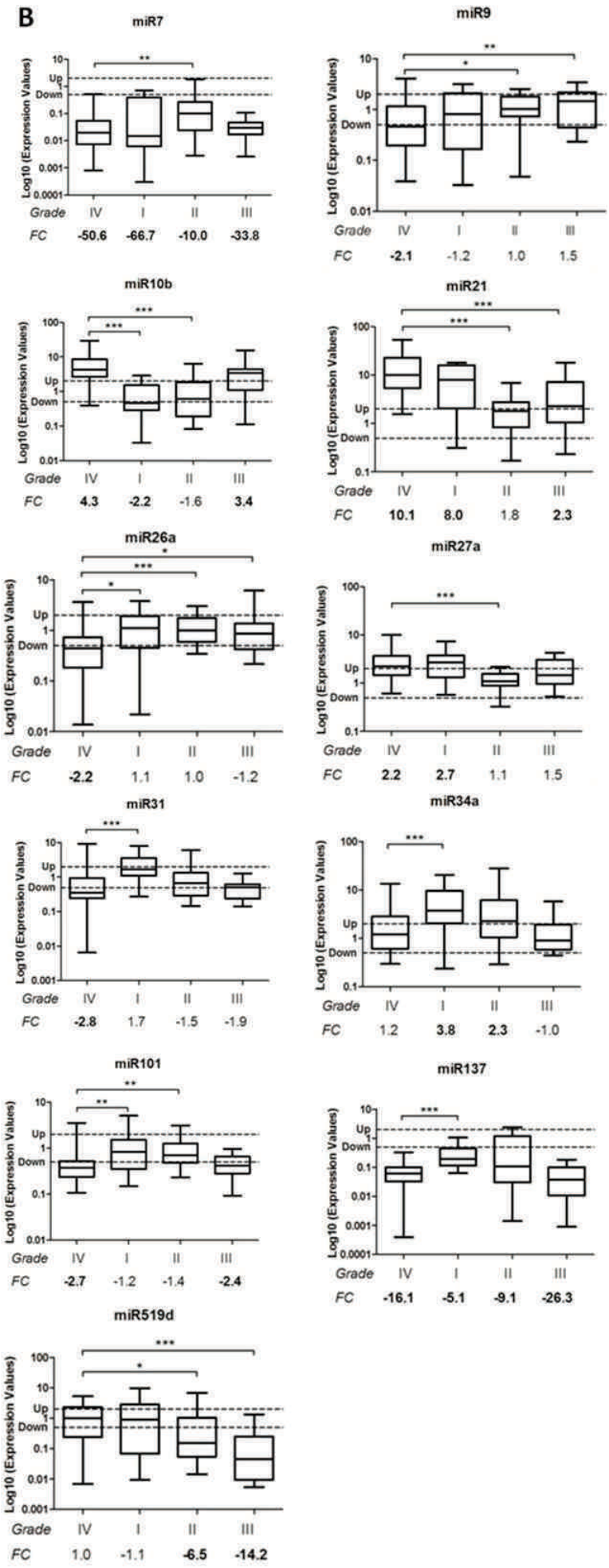

B

Appendix E

Higher magnification of Figure 4.14. Differences in miRNAs expression among the 4 tumor groups.

The analysis was performed using Ambion Commercial Reference (A) or Epileptic group (B) as controls. Box plots show microRNAs significantly different between GBM Group (Grade IV) and each one of the other 3 tumor groups. In Y-axis is indicated the microRNA expression level: “Up” and “Down” lines highlight the cut-off of 2 fold change to consider a microRNA as deregulated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ according to Mann-Whitney test. FC: Fold Change.


A
miR7
Log10 (Expression Values)
Grade IV I II III
FC -23.6 -13.2 -4.7 -15.7
miR9
Grade IV I II III
FC -2.3 -1.3 -1.0 1.4
miR10b
Grade IV I II III
FC 2.5 -3.7 -2.7 2.0
miR21
Grade IV I II III
FC 6.5 5.2 1.2 1.5
miR26a
Grade IV I II III
FC -8.6 -3.5 -3.8 -4.4
miR27a
Grade IV I II III
FC 2.1 2.5 1.0 1.4
miR31
Grade IV I II III
FC -15.2 -3.2 -8.1 -10.8
miR34a
Grade IV I II III
FC 1.1 3.5 2.1 -1.1
miR101
Grade IV I II III
FC -2.6 -1.2 -1.4 -2.3
miR137
Grade IV I II III
FC -9.1 -2.9 -5.1 -14.9
miR519d
Grade IV I II III
FC -1.6 -1.8 -10.2 -22.5


B
miR7
Log10 (Expression Values)
Grade IV I II III
FC -50.6 -66.7 -10.0 -33.8
miR9
Grade IV I II III
FC -2.1 -1.2 1.0 1.5
miR10b
Grade IV I II III
FC 4.3 -2.2 -1.6 3.4
miR21
Grade IV I II III
FC 10.1 8.0 1.8 2.3
miR26a
Grade IV I II III
FC -2.2 1.1 1.0 -1.2
miR27a
Grade IV I II III
FC 2.2 2.7 1.1 1.5
miR31
Grade IV I II III
FC -2.8 1.7 -1.5 -1.9
miR34a
Grade IV I II III
FC 1.2 3.8 2.3 -1.0
miR101
Grade IV I II III
FC -2.7 -1.2 -1.4 -2.4
miR137
Grade IV I II III
FC -16.1 -5.1 -9.1 -26.3
miR519d
Grade IV I II III
FC 1.0 -1.1 -6.5 -14.2

Bibliography

1. Dolecek, T.A., *et al.*, *CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009.* Neuro Oncol, 2012. **14 Suppl 5**: p. v1-49.
2. Huse, J.T., H.S. Phillips, and C.W. Brennan, *Molecular subclassification of diffuse gliomas: seeing order in the chaos.* Glia, 2011. **59**(8): p. 1190-9.
3. Ricard, D., *et al.*, *Primary brain tumours in adults.* Lancet, 2012. **379**(9830): p. 1984-96.
4. Louis, D.N., *et al.*, *WHO. Classification of Tumours of the Central Nervous System*, ed. IARC. 2007, Lyon: IARC.
5. Furnari, F.B., *et al.*, *Malignant astrocytic glioma: genetics, biology, and paths to treatment.* Genes Dev, 2007. **21**(21): p. 2683-710.
6. Brennan, C., *Genomic profiles of glioma.* Curr Neurol Neurosci Rep, 2011. **11**(3): p. 291-7.
7. Verhaak, R.G., *et al.*, *Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1.* Cancer Cell, 2010. **17**(1): p. 98-110.
8. Brandes, A.A., *et al.*, *Recurrence pattern after temozolomide concomitant with and adjuvant to radiotherapy in newly diagnosed patients with glioblastoma: correlation With MGMT promoter methylation status.* J Clin Oncol, 2009. **27**(8): p. 1275-9.
9. Clarke, J., N. Butowski, and S. Chang, *Recent advances in therapy for glioblastoma.* Arch Neurol, 2010. **67**(3): p. 279-83.
10. Masui, K., T.F. Cloughesy, and P.S. Mischel, *Review: molecular pathology in adult high-grade gliomas: from molecular diagnostics to target therapies.* Neuropathol Appl Neurobiol, 2012. **38**(3): p. 271-91.
11. Ermoian, R.P., *et al.*, *Dysregulation of PTEN and protein kinase B is associated with glioma histology and patient survival.* Clin Cancer Res, 2002. **8**(5): p. 1100-6.
12. Nicotera, P. and G. Melino, *Regulation of the apoptosis-necrosis switch.* Oncogene, 2004. **23**(16): p. 2757-65.
13. Stegh, A.H., *et al.*, *Bcl2L12 inhibits post-mitochondrial apoptosis signaling in glioblastoma.* Genes Dev, 2007. **21**(1): p. 98-111.
14. Bulnes, S., *et al.*, *Angiogenic signalling pathways altered in gliomas: selection mechanisms for more aggressive neoplastic subpopulations with invasive phenotype.* J Signal Transduct, 2012. **2012**: p. 597915.
15. Wick, W., *et al.*, *BCL-2 promotes migration and invasiveness of human glioma cells.* FEBS Lett, 1998. **440**(3): p. 419-24.
16. Bai, R.Y., V. Staedtke, and G.J. Riggins, *Molecular targeting of glioblastoma: Drug discovery and therapies.* Trends Mol Med, 2011. **17**(6): p. 301-12.

17. Mellinghoff, I.K., *et al.*, *Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors.* N Engl J Med, 2005. **353**(19): p. 2012-24.
18. Fan, Q.W., *et al.*, *A dual phosphoinositide-3-kinase alpha/mTOR inhibitor cooperates with blockade of epidermal growth factor receptor in PTEN-mutant glioma.* Cancer Res, 2007. **67**(17): p. 7960-5.
19. Natsume, A. and J. Yoshida, *Gene therapy for high-grade glioma: current approaches and future directions.* Cell Adh Migr, 2008. **2**(3): p. 186-91.
20. Tobias, A., *et al.*, *The art of gene therapy for glioma: a review of the challenging road to the bedside.* J Neurol Neurosurg Psychiatry, 2012. **84**(2): p. 213-22.
21. Catuogno, S., *et al.*, *Nucleic acids in human glioma treatment: innovative approaches and recent results.* J Signal Transduct, 2012. **2012**: p. 735135.
22. Yang, H., *Nanoparticle-mediated brain-specific drug delivery, imaging, and diagnosis.* Pharm Res, 2010. **27**(9): p. 1759-71.
23. Hau, P., P. Jachimczak, and U. Bogdahn, *Treatment of malignant gliomas with TGF-beta2 antisense oligonucleotides.* Expert Review Anticancer Therapy, 2009. **9**(11): p. 1163-1674.
24. Han, L., *et al.*, *Combination gene therapy with PTEN and EGFR siRNA suppresses U251 malignant glioma cell growth in vitro and in vivo.* Med Oncol, 2010. **27**(3): p. 843-52.
25. Esposito, C.L., *et al.*, *A neutralizing RNA aptamer against EGFR causes selective apoptotic cell death.* PLoS One, 2011. **6**(9): p. e24071.
26. Wong, S.T., *et al.*, *MicroRNA-21 inhibition enhances in vitro chemosensitivity of temozolomide-resistant glioblastoma cells.* Anticancer Res, 2012. **32**(7): p. 2835-41.
27. LeBrun, D.G. and M. Li, *MicroRNAs in Glioblastoma Multiforme: Profiling Studies and Therapeutic Impacts.* Mol Cell Pharmacol, 2011. **3**(3): p. 93-105.
28. Nana-Sinkam, S.P. and C.M. Croce, *Clinical applications for microRNAs in cancer.* Clin Pharmacol Ther, 2013. **93**(1): p. 98-104.
29. Zeng, Y., *Principles of micro-RNA production and maturation.* Oncogene, 2006. **25**(46): p. 6156-62.
30. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
31. Filipowicz, W., S.N. Bhattacharyya, and N. Sonenberg, *Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?* Nat Rev Genet, 2008. **9**(2): p. 102-14.

32. Lewis, B.P., C.B. Burge, and D.P. Bartel, *Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets.* Cell, 2005. **120**(1): p. 15-20.
33. Shukla, G.C., J. Singh, and S. Barik, *MicroRNAs: Processing, Maturation, Target Recognition and Regulatory Functions.* Mol Cell Pharmacol, 2011. **3**(3): p. 83-92.
34. Thomas, M., J. Lieberman, and A. Lal, *Desperately seeking microRNA targets.* Nat Struct Mol Biol, 2010. **17**(10): p. 1169-74.
35. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.
36. Bernardo, B.C., *et al.*, *A microRNA guide for clinicians and basic scientists: background and experimental techniques.* Heart Lung Circ, 2012. **21**(3): p. 131-42.
37. Wuchty, S., *et al.*, *Prediction of Associations between microRNAs and Gene Expression in Glioma Biology.* PLoS One, 2011. **6**(2): p. e14681.
38. Hua, Y.J., *et al.*, *Identification and target prediction of miRNAs specifically expressed in rat neural tissue.* BMC Genomics, 2009. **10**: p. 214.
39. Witkos, T.M., E. Koscianska, and W.J. Krzyzosiak, *Practical Aspects of microRNA Target Prediction.* Curr Mol Med, 2011. **11**(2): p. 93-109.
40. Xiao, F., *et al.*, *miRecords: an integrated resource for microRNA-target interactions.* Nucleic Acids Res, 2009. **37**(Database issue): p. D105-10.
41. Hsu, S.D., *et al.*, *miRTarBase: a database curates experimentally validated microRNA-target interactions.* Nucleic Acids Res, 2011. **39**(Database issue): p. D163-9.
42. Dweep, H., *et al.*, *miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes.* J Biomed Inform, 2011. **44**(5): p. 839-47.
43. Muniategui, A., *et al.*, *Joint analysis of miRNA and mRNA expression data.* Brief Bioinform, 2012.
44. Zhang, B., *et al.*, *microRNAs as oncogenes and tumor suppressors.* Dev Biol, 2007. **302**(1): p. 1-12.
45. Galasso, M., S.K. Sandhu, and S. Volinia, *MicroRNA expression signatures in solid malignancies.* Cancer J, 2012. **18**(3): p. 238-43.
46. Lawler, S. and E.A. Chiocca, *Emerging functions of microRNAs in glioblastoma.* J Neurooncol, 2009. **92**(3): p. 297-306.
47. Vannini, I., F. Fanini, and M. Fabbri, *MicroRNAs as lung cancer biomarkers and key players in lung carcinogenesis.* Clin Biochem, 2013.
48. Lee, E.J., *et al.*, *Expression profiling identifies microRNA signature in pancreatic cancer.* Int J Cancer, 2007. **120**(5): p. 1046-54.

49. Schaefer, A., *et al.*, *Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma.* Int J Cancer, 2010. **126**(5): p. 1166-76.
50. de la Chapelle, A. and K. Jazdzewski, *MicroRNAs in thyroid cancer.* J Clin Endocrinol Metab, 2011. **96**(11): p. 3326-36.
51. Fu, S.W., L. Chen, and Y.G. Man, *miRNA Biomarkers in Breast Cancer Detection and Management.* J Cancer, 2011. **2**: p. 116-22.
52. Voller, D., C. Ott, and A. Bosserhoff, *MicroRNAs in malignant melanoma.* Clin Biochem, 2013.
53. Schetter, A.J., *et al.*, *MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma.* JAMA, 2008. **299**(4): p. 425-36.
54. Di Lisio, L., *et al.*, *MicroRNA signatures in B-cell lymphomas.* Blood Cancer J, 2012. **2**(2): p. e57.
55. Selcuklu, S.D., M.T. Donoghue, and C. Spillane, *miR-21 as a key regulator of oncogenic processes.* Biochem Soc Trans, 2009. **37**(Pt 4): p. 918-25.
56. Tivnan, A., *et al.*, *MicroRNA-34a is a potent tumor suppressor molecule in vivo in neuroblastoma.* BMC Cancer, 2011. **11**: p. 33.
57. Chan, J.A., A.M. Krichevsky, and K.S. Kosik, *MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells.* Cancer Res, 2005. **65**(14): p. 6029-33.
58. Ciafre, S.A., *et al.*, *Extensive modulation of a set of microRNAs in primary glioblastoma.* Biochem Biophys Res Commun, 2005. **334**(4): p. 1351-8.
59. Silber, J., *et al.*, *miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells.* BMC Med, 2008. **6**: p. 14.
60. Conti, A., *et al.*, *miR-21 and 221 upregulation and miR-181b downregulation in human grade II-IV astrocytic tumors.* J Neurooncol, 2009. **93**(3): p. 325-32.
61. Gal, H., *et al.*, *MIR-451 and Imatinib mesylate inhibit tumor growth of Glioblastoma stem cells.* Biochem Biophys Res Commun, 2008. **376**(1): p. 86-90.
62. Godlewski, J., *et al.*, *Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal.* Cancer Res, 2008. **68**(22): p. 9125-30.
63. Rao, S.A., V. Santosh, and K. Somasundaram, *Genome-wide expression profiling identifies deregulated miRNAs in malignant astrocytoma.* Mod Pathol, 2010. **23**(10): p. 1404-17.
64. Slaby, O., *et al.*, *MicroRNA-181 family predicts response to concomitant chemoradiotherapy with temozolomide in glioblastoma patients.* Neoplasma, 2010. **57**(3): p. 264-9.

65. Zhou, X., *et al.*, *Downregulation of miR-21 inhibits EGFR pathway and suppresses the growth of human glioblastoma cells independent of PTEN status.* Lab Invest, 2010. **90**(2): p. 144-55.
66. Dong, H., *et al.*, *Integrated analysis of mutations, miRNA and mRNA expression in glioblastoma.* BMC Syst Biol, 2010. **4**: p. 163.
67. Guan, Y., *et al.*, *MiRNA-196 is upregulated in glioblastoma but not in anaplastic astrocytoma and has prognostic significance.* Clin Cancer Res, 2010. **16**(16): p. 4289-97.
68. Hua, D., *et al.*, *A catalogue of glioblastoma and brain MicroRNAs identified by deep sequencing.* OMICS, 2012. **16**(12): p. 690-9.
69. Lang, M.F., *et al.*, *Genome-wide profiling identified a set of miRNAs that are differentially expressed in glioblastoma stem cells and normal neural stem cells.* PLoS One, 2012. **7**(4): p. e36248.
70. Loftus, J.C., *et al.*, *miRNA expression profiling in migrating glioblastoma cells: regulation of cell migration and invasion by miR-23b via targeting of Pyk2.* PLoS One, 2012. **7**(6): p. e39818.
71. Malzkorn, B., *et al.*, *Identification and functional characterization of microRNAs involved in the malignant progression of gliomas.* Brain Pathol, 2009. **20**(3): p. 539-50.
72. Niyazi, M., *et al.*, *MiRNA expression patterns predict survival in glioblastoma.* Radiat Oncol, 2011. **6**: p. 153.
73. Sasayama, T., *et al.*, *MicroRNA-10b is overexpressed in malignant glioma and associated with tumor invasive factors, uPAR and RhoC.* Int J Cancer, 2009. **125**(6): p. 1407-13.
74. Skalsky, R.L. and B.R. Cullen, *Reduced expression of brain-enriched microRNAs in glioblastomas permits targeted regulation of a cell death gene.* PLoS One, 2011. **6**(9): p. e24248.
75. Srinivasan, S., I.R. Patric, and K. Somasundaram, *A ten-microRNA expression signature predicts survival in glioblastoma.* PLoS One, 2011. **6**(3): p. e17438.
76. Zhang, W., *et al.*, *Whole-genome microRNA expression profiling identifies a 5-microRNA signature as a prognostic biomarker in Chinese patients with primary glioblastoma multiforme.* Cancer, 2013. **119**(4): p. 814-24.
77. Chen, Y., *et al.*, *MicroRNA-21 down-regulates the expression of tumor suppressor PDCD4 in human glioblastoma cell T98G.* Cancer Lett, 2008. **272**(2): p. 197-205.
78. Huse, J.T., *et al.*, *The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo.* Genes Dev, 2009. **23**(11): p. 1327-37.

79. Kefas, B., *et al.*, *microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma.* Cancer Res, 2008. **68**(10): p. 3566-72.
80. Li, W.B., *et al.*, *MicroRNA-34a targets notch1 and inhibits cell proliferation in glioblastoma multiforme.* Cancer Biol Ther, 2011. **12**(6): p. 477-83.
81. Smits, M., *et al.*, *miR-101 is down-regulated in glioblastoma resulting in EZH2-induced proliferation, migration, and angiogenesis.* Oncotarget, 2010. **1**(8): p. 710-20.
82. Mizoguchi, M., *et al.*, *MicroRNAs in Human Malignant Gliomas.* J Oncol, 2012. **2012**: p. 732874.
83. Novakova, J., *et al.*, *MicroRNA involvement in glioblastoma pathogenesis.* Biochem Biophys Res Commun, 2009. **386**(1): p. 1-5.
84. Zhang, C., *et al.*, *Co-suppression of miR-221/222 cluster suppresses human glioma cell growth by targeting p27kip1 in vitro and in vivo.* Int J Oncol, 2009. **34**(6): p. 1653-60.
85. Zhang, C., *et al.*, *High level of miR-221/222 confers increased cell invasion and poor prognosis in glioma.* J Transl Med, 2012. **10**: p. 119.
86. Zhang, J., *et al.*, *miR-221/222 promote malignant progression of glioma through activation of the Akt pathway.* Int J Oncol, 2010. **36**(4): p. 913-20.
87. Pang, J.C., *et al.*, *Oncogenic role of microRNAs in brain tumors.* Acta Neuropathol, 2009. **117**(6): p. 599-611.
88. Karsy, M., E. Arslan, and F. Moy, *Current Progress on Understanding MicroRNAs in Glioblastoma Multiforme.* Genes Cancer, 2012. **3**(1): p. 3-15.
89. Singh, S.K., *et al.*, *A microRNA Link to Glioblastoma Heterogeneity.* Cancers, 2012. **4**: p. 846-872.
90. Feng, S.Y., *et al.*, *Lentiviral expression of anti-microRNAs targeting miR-27a inhibits proliferation and invasiveness of U87 glioma cells.* Mol Med Rep, 2012. **6**(2): p. 275-81.
91. Gabriely, G., *et al.*, *Human glioma growth is controlled by microRNA-10b.* Cancer Res, 2010. **71**(10): p. 3563-72.
92. Genovese, G., *et al.*, *microRNA regulatory network inference identifies miR-34a as a novel regulator of TGF-beta signaling in glioblastoma.* Cancer Discov, 2012. **2**(8): p. 736-49.
93. Gillies, J.K. and I.A. Lorimer, *Regulation of p27Kip1 by miRNA 221/222 in glioblastoma.* Cell Cycle, 2007. **6**(16): p. 2005-9.
94. Li, Y., *et al.*, *MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes.* Cancer Res, 2009. **69**(19): p. 7569-76.
95. Lukiw, W.J., *et al.*, *Up-regulation of micro-RNA-221 (miRNA-221; chr Xp11.3) and caspase-3 accompanies down-regulation of the survivin-1*

homolog BIRC1 (NAIP) in glioblastoma multiforme (GBM). J Neurooncol, 2009. **91**(1): p. 27-32.

96. Nass, D., *et al.*, *MiR-92b and miR-9/9* are specifically expressed in brain primary tumors and can be used to differentiate primary from metastatic brain tumors.* Brain Pathol, 2009. **19**(3): p. 375-83.
97. Quintavalle, C., *et al.*, *miR-221/222 overexpession in human glioblastoma increases invasiveness by targeting the protein phosphate PTPmu.* Oncogene, 2012. **31**(7): p. 858-68.
98. Silber, J., *et al.*, *miR-34a repression in proneural malignant gliomas upregulates expression of its target PDGFRA and promotes tumorigenesis.* PLoS One, 2012. **7**(3): p. e33844.
99. Ujifuku, K., *et al.*, *miR-195, miR-455-3p and miR-10a(*) are implicated in acquired temozolomide resistance in glioblastoma multiforme cells.* Cancer Lett, 2010. **296**(2): p. 241-8.
100. Webster, R.J., *et al.*, *Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7.* J Biol Chem, 2009. **284**(9): p. 5731-41.
101. Moller, H.G., *et al.*, *A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion.* Mol Neurobiol, 2013. **47**(1): p. 131-44.
102. Silber, J., C.D. James, and J.G. Hodgson, *microRNAs in gliomas: small regulators of a big problem.* Neuromolecular Med, 2009. **11**(3): p. 208-22.
103. Xi, Y., *et al.*, *Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples.* RNA, 2007. **13**(10): p. 1668-74.
104. Li, J., *et al.*, *Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells.* BMC Biotechnol, 2007. **7**: p. 36.
105. Winter, J. and S. Diederichs, *Argonaute proteins regulate microRNA stability: Increased microRNA abundance by Argonaute proteins is due to microRNA stabilization.* RNA Biol, 2011. **8**(6): p. 1149-57.
106. Siebolts, U., *et al.*, *Tissues from routine pathology archives are suitable for microRNA analyses by quantitative PCR.* J Clin Pathol, 2009. **62**(1): p. 84-8.
107. Nonn, L., *et al.*, *mRNA and microRNA Expression Analysis in Laser Capture Microdissected-Prostate Biopsies: Valuable tool for risk assessment and prevention trials.* Exp Mol Pathol, 2010. **88**(1).
108. Leite, K.R., *et al.*, *miRNA analysis of prostate cancer by quantitative real time PCR: comparison between formalin-fixed paraffin embedded and fresh-frozen tissue.* Urol Oncol, 2009. **29**(5): p. 533-7.

109. Goswami, R.S., *et al.*, *Optimization and analysis of a quantitative real-time PCR-based technique to determine microRNA expression in formalin-fixed paraffin-embedded samples.* BMC Biotechnol, 2010. **10**: p. 47.
110. Zhang, X., *et al.*, *An array-based analysis of microRNA expression comparing matched frozen and formalin-fixed paraffin-embedded human tissue samples.* J Mol Diagn, 2008. **10**(6): p. 513-9.
111. Hasemeier, B., *et al.*, *Reliable microRNA profiling in routinely processed formalin-fixed paraffin-embedded breast cancer specimens using fluorescence labelled bead technology.* BMC Biotechnol, 2008. **8**: p. 90.
112. van Rooij, E., *The art of microRNA research.* Circ Res, 2011. **108**(2): p. 219-34.
113. Roa, W., *et al.*, *Identification of a new microRNA expression profile as a potential cancer screening tool.* Clin Invest Med, 2010. **33**(2): p. E124.
114. Quintavalle, C., *et al.*, *miR-221/222 overexpession in human glioblastoma increases invasiveness by targeting the protein phosphate PTPmu.* Oncogene, 2011. **31**(7): p. 858-68.
115. Morandi, L., *et al.*, *Promoter methylation analysis of O6-methylguanine-DNA methyltransferase in glioblastoma: detection by locked nucleic acid based quantitative PCR using an imprinted gene (SNURF) as a reference.* BMC Cancer, 2010. **10**: p. 48.
116. Morandi, L., *et al.*, *Allele specific locked nucleic acid quantitative PCR (ASLNAqPCR): an accurate and cost-effective assay to diagnose and quantify KRAS and BRAF mutation.* PLoS One, 2012. **7**(4): p. e36084.
117. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
118. Cho, R.J. and M.J. Campbell, *Transcription, genomes, function.* Trends Genet, 2000. **16**(9): p. 409-15.
119. Yan, H., *et al.*, *IDH1 and IDH2 mutations in gliomas.* N Engl J Med, 2009. **360**(8): p. 765-73.
120. de Biase, D., *et al.*, *miRNAs expression analysis in paired fresh/frozen and dissected formalin fixed and paraffin embedded glioblastoma using real-time pCR.* PLoS One, 2012. **7**(4): p. e35596.
121. Dong, H., *et al.*, *Investigation gene and microRNA expression in glioblastoma.* BMC Genomics, 2010. **11 Suppl 3**: p. S16.
122. Visani, M., *et al.*, *Definition of miRNAs Expression Profile in Glioblastoma Samples: The Relevance of Non-Neoplastic Brain Reference.* PLoS One, 2012. **8**(1): p. e55314.
123. Nakada, M., *et al.*, *Aberrant Signaling Pathways in Glioma.* Cancers, 2011. **3**: p. 3242-3278.

124. de la Iglesia, N., *et al.*, *Deregulation of a STAT3-interleukin 8 signaling pathway promotes human glioblastoma cell proliferation and invasiveness.* J Neurosci, 2008. **28**(23): p. 5870-8.
125. Low, S., *et al.*, *Pathogenetic pathways leading to glioblastoma multiforme: association between gene expressions and resistance to erlotinib.* Anticancer Res, 2008. **28**(6A): p. 3729-32.
126. Montagnani Marelli, M., *et al.*, *Novel insights into GnRH receptor activity: role in the control of human glioblastoma cell proliferation.* Oncol Rep, 2009. **21**(5): p. 1277-82.
127. Trojan, J., *et al.*, *Insulin-like growth factor type I biology and targeting in malignant gliomas.* Neuroscience, 2007. **145**(3): p. 795-811.
128. van Groeninghen, J.C., *et al.*, *Effects of luteinising-hormone-releasing hormone on nervous-system tumours.* Lancet, 1998. **352**(9125): p. 372-3.
129. Wang, L., *et al.*, *Interleukin-1beta and transforming growth factor-beta cooperate to induce neurosphere formation and increase tumorigenicity of adherent LN-229 glioma cells.* Stem Cell Res Ther, 2012. **3**(1): p. 5.

Ringraziamenti

Prima di tutto vorrei ringraziare la Professoressa Annalisa Pession, per avermi permesso di frequentare il suo laboratorio dal lontano 2006 ad oggi. A lei devo la mia carriera universitaria, dalla laurea triennale fino a questo dottorato: grazie per i continui insegnamenti, per la disponibilità e la fiducia dimostratami.

A Dario un grazie speciale, per tutto: per avermi trasmesso l'amore e la passione per questo lavoro, per l'aiuto, la disponibilità e l'infinita pazienza.

Un grande ringraziamento a tutta l'Anatomia Patologica dell'Ospedale Bellaria: ho conosciuto persone veramente valide, che non dimenticherò mai.

A Serena, Giorgia, Paola, Federica, Sara e Valentina, chi da più e chi da meno tempo, per essere presenti nella mia vita e per avere condiviso con me bellissimi momenti in questi ultimi 3 anni.

Un immenso grazie a Cinzia&Andrea, non avrei mai potuto desiderare genitori migliori: grazie per avermi permesso di arrivare fino a qui, di inseguire una passione che senza il vostro aiuto e appoggio costante, si sarebbe spenta sul nascere.

L'ultimo grazie corre a nonno: perché nessuno è mai stato tanto fiero di me e perché avrebbe sfogliato e spulciato ogni riga di questa tesi, pur non sapendo l'inglese. Conseguire questo titolo senza vederlo tra il pubblico non sarà lo stesso, ma sarà con me in ogni istante.

OPEN ACCESS Freely available online

PLoS one

miRNAs Expression Analysis in Paired Fresh/Frozen and Dissected Formalin Fixed and Paraffin Embedded Glioblastoma Using Real-Time PCR

Dario de Biase[1,2*☯], Michela Visani[1☯], Luca Morandi[2], Gianluca Marucci[2], Cristian Taccioli[3], Serenella Cerasoli[4], Agostino Baruzzi[5], Annalisa Pession[1], the PERNO Study group[¶]

1 Department of Patologia Sperimentale, University of Bologna, Bologna, Italy, **2** Department of Ematologia e Scienze Oncologiche, University of Bologna, Bologna, Italy, **3** Department of Cancer Biology, Paul O'Gorman Cancer Institute, University College London, London, United Kingdom, **4** Anatomic Pathology of Bufalini Hospital, Cesena, Italy, **5** IRCCS Istituto delle Scienze Neurologiche di Bologna and Department of Biomedical & Neuromotor Sciences, University of Bologna, Bologna, Italy

Abstract

miRNAs are small molecules involved in gene regulation. Each tissue shows a characteristic miRNAs epression profile that could be altered during neoplastic transformation. Glioblastoma is the most aggressive brain tumour of the adult with a high rate of mortality. Recognizing a specific pattern of miRNAs for GBM could provide further boost for target therapy. The availability of fresh tissue for brain specimens is often limited and for this reason the possibility of starting from formalin fixed and paraffin embedded tissue (FFPE) could very helpful even in miRNAs expression analysis. We analysed a panel of 19 miRNAs in 30 paired samples starting both from FFPE and Fresh/Frozen material. Our data revealed that there is a good correlation in results obtained from FFPE in comparison with those obtained analysing miRNAs extracted from Fresh/Frozen specimen. In the few cases with a not good correlation value we noticed that the discrepancy could be due to dissection performed in FFPE samples. To the best of our knowledge this is the first paper demonstrating that the results obtained in miRNAs analysis using Real-Time PCR starting from FFPE specimens of glioblastoma are comparable with those obtained in Fresh/Frozen samples.

Citation: de Biase D, Visani M, Morandi L, Marucci G, Taccioli C, et al. (2012) miRNAs Expression Analysis in Paired Fresh/Frozen and Dissected Formalin Fixed and Paraffin Embedded Glioblastoma Using Real-Time PCR. PLoS ONE 7(4): e35596. doi:10.1371/journal.pone.0035596

Editor: Svetlana Kotliarova, NIH/NCI, United States of America

Received March 13, 2012; **Accepted** March 20, 2012; **Published** April 18, 2012

Funding: This study was funded by Progetto Emiliano-Romagnolo di Neuro-Oncologia (PERNO) (Emilia-Romagna region project). Dr. de Biase was funded by C.I.R.C. (Centro Interdipartimentale di Ricerche sul Cancro 'G.Prodi', University of Bologna). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: dario.debiase@unibo.it

☯ These authors contributed equally to this work.
¶ Membership of the PERNO Study Group is provided in the Acknowledgments.

Introduction

MicroRNAs (or miRNAs) are small (~20–22 nt) non coding RNAs that modulate gene expression at a post-transcriptional level. They act by binding the target mRNAs repressing translation or regulating their degradation. Each miRNA, playing its role through perfect and nearly perfect complementarity with its target mRNAs, could regulate the expression of about a hundred of genes, influencing a large spectrum of physiological processes as different steps of cellular development, proliferation or apoptosis regulation [1].

Many of these pathways are altered in human neoplasia; in fact it has been demonstrated that miRNAs can act both as oncogenes or oncosuppressors, according to their target mRNAs [2]. In fact, in several neoplasia it has been observed that physiological miRNAs profile resulted modified [3–7].

Glioblastoma (GBM) is a highly malignant astrocytic glioma. It is the most frequent primary brain tumour and the most malignant neoplasm with astrocytic differentiation and correspond to WHO grade IV [8]. Histologically it is composed of poorly differentiated astrocytic tumour cells, with marked nuclear atypia, high mitotic activity, prominent microvascular proliferation and necrosis. Neverthless the progress in neurosurgery, chemio- and radiotherapy, molecular target identification for focused therapy (MGMT), the clinical history of the disease is usually short (less than one year in more than 50% of cases) [8,9].

There are several evidences that different miRNAs could be up- or down-regulated in GBM. MiR-9/9* [10–12], miR-10a [13], miR10b [12,14–16], miR17 [11], miR20a [11], miR-21 [11,12,14,16,17], miR26 [18], miR27a [18], miR182 [18,19], miR-221 [12,20–22], miR-222 [22] and miR-519d [16] were observed to be up-regulated in GBM (Table 1); on the contrary miR-7 [14,23–25], miR-31 [14], miR34a [26,27], miR-101 [14,28], miR-137 [14,16], miR-330 [14] were recognized as down-regulated (Table 1). The increasing evidence that miRNAs are involved in GBM development and progression could lead to recognise a specific miRNAs profile for this neoplasia.

It has been demonstrated that, differently from mRNA, integrity of miRNAs is not influenced by fixation in formalin [29], probably due to their short length and to the complex Argonaute protein-miRNA [30]. The comparison of miRNAs expression starting from Fresh/Frozen or FFPE (formalin fixed and paraffin

Table 1. Name, chromosomal localization and expression level in GBM according to previously described data of miRNAs analysed in this study.

miRNA	Localization	Up/Downregulated in GBM	Reference
9/9*	1q22	UP	[10–12]
10a	17q21.32	UP	[13]
10b	2q31.1	UP	[12,14–16]
17	13q31.3	UP	[11]
20a	13q31.3	UP	[11]
21	17q21.31	UP	[11,12,14,16,17]
26	3p22.2	UP	[18]
27a	19p13.13	UP	[18]
182	7q32.2	UP	[18,19]
221	Xp11.3	UP	[12,20–22]
222	Xp11.3	UP	[22]
519d	19q13.42	UP	[16]
7	9q21.3	DOWN	[14,23–25]
31	9p21.3	DOWN	[14]
34a	1p36.22	DOWN	[26,27]
101	1p31.3	DOWN	[14,28]
137	1p21.3	DOWN	[14,16]
330	19q13.32	DOWN	[14]

doi:10.1371/journal.pone.0035596.t001

embedded) material was performed in culture cells [31] and in several tissues as prostate [32,33], breast [34–36], kidney [29,37,38], lymphatic tissue [39,40], tonsils [37], melanocytic nevi [41], colon carcinoma [38] and in one case of oligodendroglioma [42]. All these papers have demonstrated that there was a good correlation in miRNAs expression analysis starting both Fresh/Frozen and FFPE tissue. None of them, except for Nonn et al. [32], performed dissection in Fresh/Frozen or FFPE material. Most of miRNAs expression studies in GBM were performed on Fresh/Frozen tissue or cell lines. In central nervous system neoplasia, starting from FFPE tissue could be very useful because of archival material is readily available and follow-up is often known.

Aim of this study was to investigate the expression of 19 miRNAs in GBM starting from both Fresh/Frozen and FFPE-dissected tissues. In these last samples, the dissection allowed to enrich (>90%) the analysed material of neoplastic cells, limiting the eventual contamination due to "normal near the tumour" fraction (e.g. lymphocytes, stroma, not neoplastic glial and neuronal cells). In this way we would to investigate the feasibility of miRNAs expression analysis starting from FFPE tissues in GBM, looking for eventually differences between not dissected Fresh/Frozen samples and FFPE-dissected tissues.

Materials and Methods

Ethic Statement

The study was approved by Ethic Committee of Azienda Sanitaria Locale di Bologna (number of study 08075, protocol number 139/CE of 5th February 2009, Bologna, Italy). All patients signed a written consent for molecular analysis and for anonymous data publication for scientific studies and all information regarding the human material used in this study was managed using anonymous numerical codes.

Selection of Cases

Thirty cases of GBM were selected for miRNAs expression analysis from cases collected at Bellaria (institute of Anatomia Patologica, Bologna, Italy) and Bufalini (institute of Anatomia Patologica, Cesena, Italy) Hospitals, within PERNO (Progetto Emiliano-Romagnolo di Neuro-Oncologia) project. All specimens were primary GBM, and patients had not undergone neoadjuvant therapy before surgery. Patients were 14 males and 16 females, aged from 42 to 75 years (mean 63.3 ys).

The specimens were collected no longer than 45 minutes after removal and immediately a snap-frozen section was performed and the material evaluated by a pathologist in order to verify if the tissue was represented by a "high-grade glioma".

A sample of tissue was then incubated in RNA later solution (Applied Biosystem, Austin, TX, U.S.A.) for 1 hour at room temperature and stored at −80°C after quick-frozen in liquid nitrogen. The remaining specular tissue was formalin fixed and paraffin embedded for routine histological diagnosis. All 30 samples were diagnosed as GBM according the 2007 WHO criteria [8].

Cell lines of prostate carcinoma (LNCaP, CRL-1740), breast adenocarcinoma (MCF7, HTB-22) and glioblastoma (U-87 MG, HTB14), provided by American Type Culture Collection (ATCC, Rockville, MD, USA), were used for evaluating efficiency of primers per each miRNA analysed.

miRNAs extraction

The "Fresh/Frozen" specimens and cell lines were processed for miRNAs extraction protocol using *mir*Vana miRNA isolation

Table 2. Name, localization and forward primer sequence of analysed miRNAs.

miRNA	Fw Primer Sequence
hsa-miR-7	TGGAA**G**ACTA**G**TGATTTTGTT
hsa-miR-9	TCTTTG**G**TTATCTAG**C**TGTATG
hsa-miR-9*	ATAAAG**C**TA**G**ATAA**C**CGAAAG
hsa-miR-10a	ACC**C**TGTAGA**T**CCGAATTTG
hsa-miR-10b	ACC**C**TGTAGA**A**CCGAATTTG
hsa-miR-17	CAAAGTGCTTA**C**A**G**TGCAG
hsa-miR-20a	TAAAGTGCTTA**T**A**G**TGCAG
hsa-miR-21	TAG**C**TTATCA**G**ACTGATGTTG
hsa-miR-26a	CAAGTAAT**C**CAGGATA**G**GC
hsa-miR-27a	TTCA**C**AGTGGCTAAGTTC**C**
hsa-miR-31	AGGCAA**G**ATGCTGG**C**ATA
hsa-miR-34a	TGG**C**AGTGT**C**TTAGCTG
hsa-miR-101	TACAGTA**C**TGT**G**ATAACTGAA
hsa-miR-137	TTATTG**C**TTAAGAATA**C**GCGT
hsa-miR-182	TTTGGCA**A**TGGTAGAA**C**TCAC
hsa-miR-221	GCTA**C**ATTGTCTG**C**TGGGTT
hsa-miR-222	GCTA**C**ATCTGG**C**TACTGG
hsa-miR-330	TCT**C**TGGGCCTGTGT**C**TTA
hsa-miR-519d	AAGTGC**C**TCC**C**TT**T**AGAGT

LNA bases are underlined. Fw: forward. Hsa: Homo sapiens (human).

doi:10.1371/journal.pone.0035596.t002

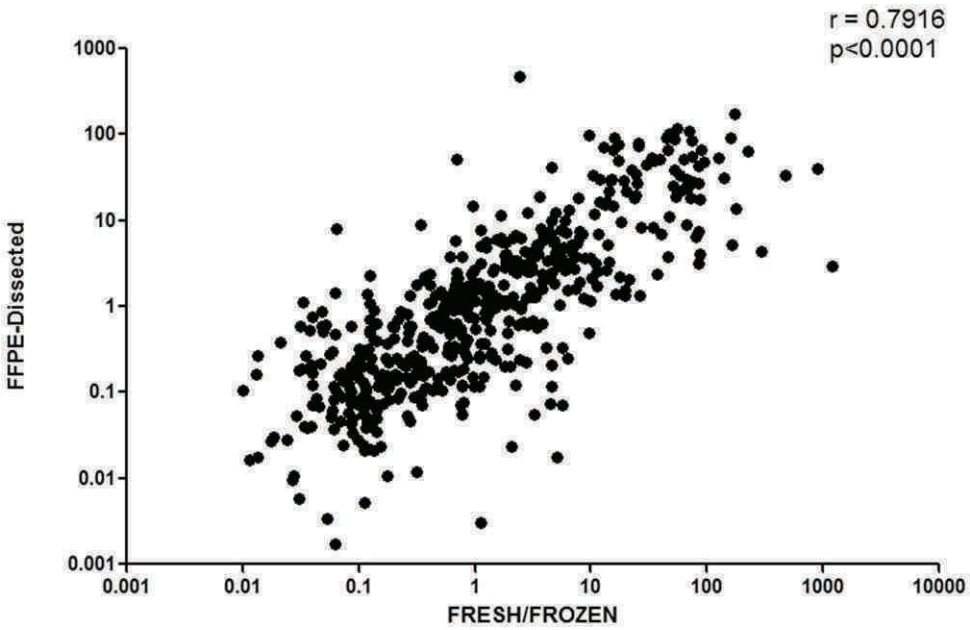


Figure 1. Scatter plot showing Spearman correlation between Fresh/Frozen and FFPE-dissected groups.
doi:10.1371/journal.pone.0035596.g001

kit (Applied Biosystem, Austin, TX, U.S.A.). Briefly, small RNA fraction was exctracted and enriched starting from 50 to 80 mg of tissue or 3 millions of cells according to manufacturer's protocol.

The haematoxylin and eosin (H&E) sections from FFPE specimens were reviewed by a pathologist (GM) to select the more informative block. Four 20 µm-thick sections were cut followed by one H&E control slide. The tumour area selected for the analysis was marked on the control slide to ensure, whenever possible, greater than 90% content of neoplastic cells (avoiding necrosis and lymphocytes). The four 20 µm-thick sections were manually dissected under microscopic guidance according to area selected on H&E and incubated in xylene for 3 minutes at 50°C and, after two rinses with ethanol, miRNAs were extracted using RecoverAll Total Nucleic Acid Isolation kit (Ambion, Austin, TX, U.S.A.), according to manufacturer's instructions.

Quality and quantity of smallRNAs extracted from both Fresh/Frozen and FFPE-dissected tissue were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and the Qubit fluorometer (Invitrogen, Carlsbad, CA, U.S.A.).

cDNA was obtained after a polyadenylation step and retro-transcription were performed using SuperScript III RT enzyme and a Universal RT Primer according to NCode miRNA first-strand cDNA synthesis and qRT-PCR Kit protocol (Invitrogen, Carlsbad, CA, U.S.A.).

miRNAs analysis

Nineteen miRNAs (Table 1) were selected for analysis, according to their role in cancer and data previously published in literature at beginning of the study [10–12,14,16–18,20,21,24,25,27]. miR103, RNU49 and U54 were used as endogenous controls.

Each forward primers used correspond to mature miRNA sequence according to miRBase database (http://microrna.sanger.ac.uk) (Table 2). Primers were modified with LNA (Locked

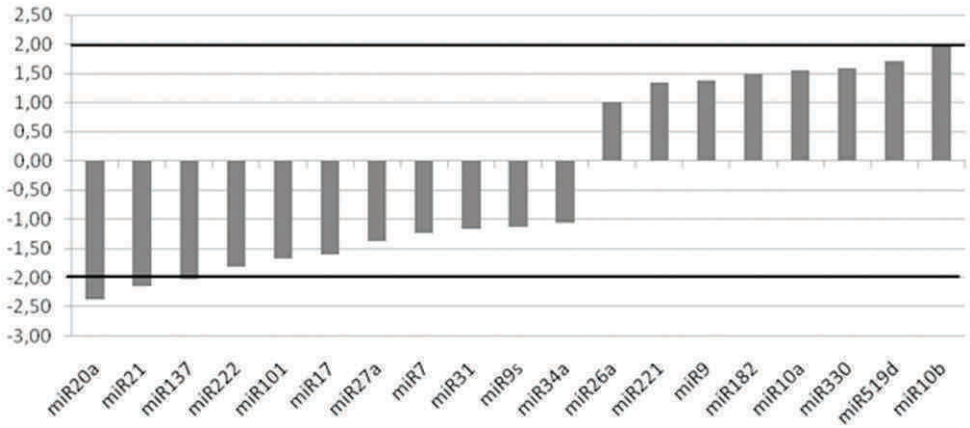


Figure 2. Median fold-change calculated per each miRNA between 30 paired Fresh/Frozen and FFPE samples. The y-axis represents the fold-change value.
doi:10.1371/journal.pone.0035596.g002

Nucleic Acid) substitutions for increasing specificity and discriminating between miRNAs with a single base different nucleotide sequences (e.g. miR-10a and miR-10b, Table 2). Universal reverse primer was provided by NCode miRNA first-strand cDNA synthesis and qRT-PCR Kit (Invitrogen, Carlsbad, CA, U.S.A.).

Efficiency of each primer was tested by Real-Time PCR using serial dilutions (1:1, 1:25, 1:50, 1:100) of a pool of RNA extracted by following cell lines: U-87 MG, MCF7 and LNCaP. A run of Real-Time PCR using as template a pool of female DNA (Promega, Madison, WI, U.S.A.) was performed to confirm that miRNAs primers were not able to amplify DNA.

miRNAs expression was evaluated using a AB7000 machine (Applied Biosystem, Foster City, CA, USA) and FastStart Taq Reagents Kit (Roche, Mannheim, Germany), with the following program: 2 minutes at 50°C, 4 minutes at 95°C and 37 cycles with annealing at 60°C for 30 seconds. GelStar stain (Lonza Bioscience, Rockland, ME, USA) was used as Real-Time detector. No template control for each miRNA was included in the reaction plate. All the reactions were performed in duplicate and amplicons run on a 3% agarose gel.

Statistical analysis

Expression values and fold-change were obtained by relative quantification and $2^{-\Delta\Delta Ct}$ method [43], using DataAssist 2.0 Tool (Applied Biosystem, Foster City, CA, USA). Statistical analysis of miRNAs expression was performed using GraphPad Prism 5.0 tool. Paired samples comparison and correlation analysis between miRNAs expression in Fresh/Frozen and FFPE-dissected samples were performed using Wilcoxon paired test and Spearman

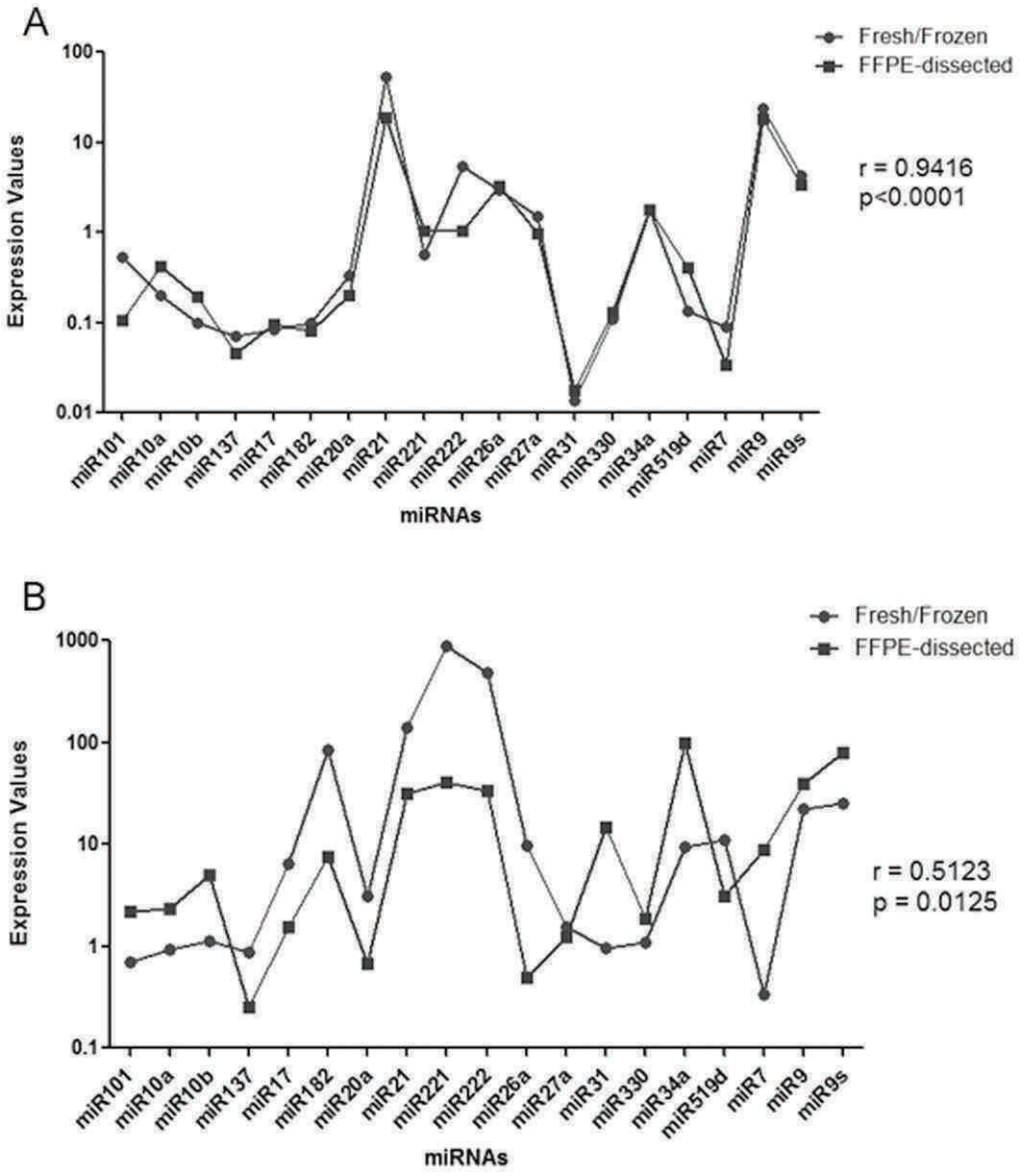


Figure 3. Comparison between miRNAs expression profile in Fresh/Frozen and in FFPE specimens. a) Example of one specimen with a good correlation of miRNAs expression profile obtained in Fresh/Frozen (pointed line) and in FFPE specimen (squared line); b) Example of one specimen with a correlation less than $r<0.65$ of miRNAs expression profile obtained in Fresh/Frozen (pointed line) and in FFPE specimen (squared line).
doi:10.1371/journal.pone.0035596.g003

Table 3. Spearman correlation values between miRNA profiles obtained in Fresh/Frozen, FFPE-dissected and FFPE-not dissected samples.

Case	R (Fresh/Frozen vs FFPE-dissected)	R (Fresh/Frozen vs FFPE-not dissected)	Composition of not-dissected FFPE sample	
			Neoplastic cells (%)	Not-neoplastic cells (%)
1	0.51	NP	98	2
2	0.60	0.70	50	50
3	0.50	0.38	75	25
4	0.62	0.81	70	30
5	0.63	0.89	40	60

NP: Not Performed.
doi:10.1371/journal.pone.0035596.t003

correlation respectively. Level of significance was $p<0.05$ for all the statistical analysis.

Results

Distribution for Fresh/Frozen (FF) and FFPE samples was found not normal, as demonstrated by the Shapiro Test ($p<0.001$). For this reason, we only used non-parametric statistical tests.

A good Spearman correlation value ($r = 0.7916$, $p<0.0001$) between the expression level of each miRNAs comparing results obtained in fresh-frozen and in FFPE-dissected samples was observed (Figure 1) whereas Wilcoxon paired test showed not significant differences between the two groups ($p = 0.1845$).

To test if the miRNAs profile obtained in Fresh/Frozen and FFPE-dissected (FD) samples were comparable, we calculated the median fold-change for each 30 FF samples versus 30 FD specimens. Although miR-137, miR-20a and miR-21 were slightly downregulated (FD/FF ratio <-2.0), the vast majority of miRNAs were not statistically significantly different (Figure 2)

Comparison of individual miRNAs expression between Fresh/Frozen and FFPE-dissected sample, in single paired specimen, showed a good Spearman correlation value ($r>0.65$) in 25 out of 30 samples (Figure 3a) while the remaining five cases showed a correlation ratio <0.65 (ranged from 0.5123 to 0.6386, Figure 3b).

To investigate if discrepancy observed in the 5 cases with $r<0.65$ could be caused by enrichment in neoplastic cells due to dissection, we analysed the miRNAs profiles of these 5 samples starting from undissected FFPE material. We performed the analysis only in the 4 cases in which the H&E revealed the presence of not-neoplastic tissue adjacent the area dissected for miRNAs analysis (Table 3). In 3 out of 4 cases analysed the Spearman correlation value increased up the cut off of 0.65 (Table 3).

Discussion

The use of formalin-fixed paraffin embedded samples for nucleic acid analysis in molecular study gives more disposal of specimen for research. For this reason, miRNAs analysis starting from FFPE samples could be of great usefulness for miRNAs expression study. Due to their short length (19–25 nt), the mature miRNAs seem not to be influenced by nucleic acid degradation caused by formalin fixation [29], as happened on the contrary for long RNA or DNA. Several papers reported the feasibility of miRNAs expression from FFPE specimens in different tissues as kidney, prostate and breast [32,33,35–38].

GBM is the most aggressive adult brain tumour and, nevertheless the progresses in molecular therapy, its prognosis remains very poor [8]. Identifying a miRNAs profile for GBM could be very useful for better clarify prognosis and researching new targeted drugs. For this reason, and for "opening" the anatomic pathology archives even to analysis of miRNAs expression in GBM, it is crucial determining if FFPE specimens are suitable for this type of analysis.

Our study demonstrated, in a cohort of 30 paired GBM, that miRNAs analysis using real-time technique could be performed starting from FFPE samples as well as from Fresh/Frozen specimens. The data demonstrated that there is a good correlation ($r = 0.7916$) between the profiles obtained starting from FFPE-dissected samples and from fresh samples.

The real cellular composition of Fresh/Frozen sample is not well known, in fact, even if a 4 μm-thick snap-frozen section was used for evaluating fresh sample, the miRNAs extraction was performed starting from 50–80 mg of not morphologically checked tissue (containing, for example, lymphocytes or non-neoplastic cells). This situation could lead to discrepant results in miRNAs analysis that we observed in 5 out of 30 cases here analysed. In FFPE-dissected samples, the selection of area used for performing the analysis lead to enrich the sample in neoplastic cells, avoiding "contamination" due to non-tumoural components. In 3 out of 4 cases, with a not good ($r<0.65$) Spearman correlation value, the analysis of miRNAs expression performed without dissection resulted in a better correlation with corresponding Fresh/Frozen samples. In only one case the correlation coefficient value remained below 0.65, even when obtained without dissecting the sample. To our knowledge, this sample did not show peculiar histological features (i.e. predominant lymphocytic infiltrate or necrotic zone).

To the best of our knowledge, this is the first study comparing the miRNAs expression analysis in GBM in FFPE-dissected samples and Fresh/Frozen specimens.

Our data demonstrated that in a cohort of 30 GBM, as happened in other tissues, data of miRNAs expression analysis are comparable starting from FFPE sample as well as from Fresh/Frozen specimens. This approach have several advantages: it is possible to check the real composition of the analysed sample, and it could be possible to dispose of archival material for miRNAs expression analysis (even considering the difficult to retrieve fresh brain tissue). The fact that dissection could influence the expression results leads to put a lot of attention in comparing miRNAs analysis performed with or without dissection.

Acknowledgments

We would like to thank Dr. Nigrisoli Evandro from Anatomic Pathology of Bufalini Hospital for kindly supplying cases. The authors thank Dr. Warren Emmett from Paul O'Gorman Cancer Institute, University College London, London, for his valuable help in the proof reading of the manuscript.

The PERNO Study group (Affiliations are indicated between brackets)

Steering committee:

Baruzzi A. (**Chair**), Albani F., Calbucci F., D'Alessandro R., Michelucci R. (IRCCS Institute of Neurological Sciences, Bologna, Italy), Brandes A. (Department of Medical Oncology, Bellaria-Maggiore Hospitals, Bologna, Italy), Eusebi V. (Department of Hematology and Oncological Sciences "L. & A. Seragnoli," Section of Anatomic Pathology at Bellaria Hospital, Bologna, Italy), Ceruti S., Fainardi E., Tamarozzi R. (Neuroradiology Unit, Department of Neurosciences and Rehabilitation, S. Anna Hospital, Ferrara, Italy), Emiliani E. (Istituto Oncologico Romagnolo, Department of Medical Oncology, Santa Maria delle Croci Hospital, Ravenna, Italy), Cavallo M. (Division of Neurosurgery, Department of Neurosciences and Rehabilitation, S. Anna Hospital, Ferrara, Italy).

Executive committee:

Franceschi E., Tosoni A. (Department of Medical Oncology, Bellaria-Maggiore Hospitals, Bologna, Italy), Cavallo M. (Division of Neurosurgery, Department of Neurosciences and Rehabilitation, S. Anna Hospital, Ferrara, Italy), Fiorica F. (Department of Radiation Oncology, S. Anna Hospital, Ferrara, Italy), Valentini A. (Division of Neurosurgery, Nuovo Ospedale Civile S. Agostino-Estense, Baggiovara, Modena, Italy), Depenni R. (Department of Oncology, Policlinico di Modena, Italy), Mucciarini C. (Department of Oncology, Ramazzini Hospital, Carpi, Modena, Italy), Crisi G. (Department of Neuroradiology, Maggiore Hospital, Parma, Italy), Sasso E. (Department of Neurological Sciences, Maggiore Hospital, Parma, Italy), Biasini C., Cavanna L. (Department of Oncology and Hematology, Guglielmo da Saliceto Hospital, Piacenza, Italy), Guidetti D. (Department of Neurology, Guglielmo da Saliceto Hospital, Piacenza, Italy), Marcello N., Pisanello A. (Department of Neurology, Istituto in tecnologie avanzate e modelli assistenziali in oncologia, IRCCS, S. Maria Nuova Hospital, Reggio Emilia, Italy), Cremonini A.M., Guiducci G. (Division of Neurosurgery, M. Bufalini Hospital, Cesena, Italy).

Registry Coordination Office: de Pasqua S., Testoni S. (IRCCS Institute of Neurological Sciences, Bologna, Italy).

Participants:

Agati R., Ambrosetto G., Bacci A., Baldin E., Baldrati A., Barbieri E., Bartolini S., Bellavista E., Bisulli F., Bonora E., Bunkheila F., Carelli V., Crisci M., Dall'Occa P., Ferro S., Franceschi C., Frezza G., GrassoV., Leonardi M., Mostacci B., Palandri G., Pasini E., Pastore Trossello M., Poggi R, Riguzzi P., Rinaldi R., Rizzi S., Romeo G., Spagnolli F., Tinuper P., Trocino C. (Bologna), Dall'Agata M., Faedi M., Frattarelli M., Gentili G., Giovannini A., Iorio P., Pasquini U., Galletti G., Guidi C., Neri W., Patuelli A., Strumia S. (Forlì-Cesena), Casmiro M., Gamboni.A., Rasi F. (Faenza, RA), Cruciani G. (Lugo, RA), Cenni P., Dazzi C., Guidi AR., Zumaglini F. (Ravenna), Amadori A., Pasini G., Pasquinelli M., Pasquini E., Polselli A., Ravasio A., Viti B. (Rimini), Sintini M. (Cattolica, RN), Ariatti A., Bertolini F., Bigliardi G., Carpeggiani P., Cavalleri F., Meletti S., Nichelli P., Pettorelli E., Pinna G., Zunarelli E. (Modena), Artioli F., Bernardini I., Costa M., Greco G., Guerzoni R., Stucchi C. (Carpi, MO), Iaccarino C., Ragazzi M., Rizzi R., Zuccoli G. (Reggio Emilia), Api P., Cartei F., Fallica E., Granieri E., Latini F., Lelli G., Monetti C., Saletti A., Schivalocchi R., Seraceni S., Tola M.R., Urbini B. (Ferrara), Giorgi C. Montanari E. (Fidenza, PR), Cerasti D., Crafa P., Dascola I., Florindo I., Giombelli E., Mazza S., Ramponi V., Servadei F., Silini EM., Torelli P. (Parma), Immovilli P., Morelli N., Vanzo C. (Piacenza), Nobile C. (Padova).

Full affiliations and postal addresses of PERNO participants are available at the study website: www.perno.it.

Author Contributions

Conceived and designed the experiments: DDB MV AP. Performed the experiments: DDB MV. Analyzed the data: DDB MV LM GM CT SC AB AP. Contributed reagents/materials/analysis tools: AP AB. Wrote the paper: DDB MV AP. Statistical Analysis: CT.

References

1. Ambros V (2004) The functions of animal microRNAs. Nature 431: 350–355.
2. Dalmay T (2008) MicroRNAs and cancer. J Intern Med 263: 366–375.
3. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, et al. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65: 7065–7070.
4. Michael MZ, SM OC, van Holst Pellekaan NG, Young GP, James RJ (2003) Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 1: 882–891.
5. Carlsson J, Davidsson S, Helenius G, Karlsson M, Lubovac Z, et al. (2011) A miRNA expression signature that separates between normal and malignant prostate tissues. Cancer Cell Int 11: 14.
6. Sozzi G, Pastorino U, Croce CM (2011) MicroRNAs and lung cancer: from markers to targets. Cell Cycle 10: 2045–2046.
7. Pang JC, Kwok WK, Chen Z, Ng HK (2009) Oncogenic role of microRNAs in brain tumors. Acta Neuropathol 117: 599–611.
8. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK (2007) WHO Classification of Tumours of the Central Nervous System; IARC, editor. Lyon.
9. Henriksson R, Asklund T, Poulsen HS (2011) Impact of therapy on quality of life, neurocognitive function and their correlates in glioblastoma multiforme: a review. J Neurooncol 104: 639–646.
10. Nass D, Rosenwald S, Meiri E, Gilad S, Tabibian-Keissar H, et al. (2009) MiR-92b and miR-9/9* are specifically expressed in brain primary tumors and can be used to differentiate primary from metastatic brain tumors. Brain Pathol 19: 375–383.
11. Malzkorn B, Wolter M, Liesenberg F, Grzendowski M, Stuhler K, et al. (2009) Identification and functional characterization of microRNAs involved in the malignant progression of gliomas. Brain Pathol 20: 539–550.
12. Ciafre SA, Galardi S, Mangiola A, Ferracin M, Liu CG, et al. (2005) Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem Biophys Res Commun 334: 1351–1358.
13. Ujifuku K, Mitsutake N, Takakura S, Matsuse M, Saenko V, et al. (2010) miR-195, miR-455-3p and miR-10a(*) are implicated in acquired temozolomide resistance in glioblastoma multiforme cells. Cancer Lett 296: 241–248.
14. Silber J, Lim DA, Petritsch C, Persson AI, Maunakea AK, et al. (2008) miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. BMC Med 6: 14.
15. Gabriely G, Yi M, Narayan RS, Niers JM, Wurdinger T, et al. (2010) Human glioma growth is controlled by microRNA-10b. Cancer Res 71: 3563–3572.
16. Godlewski J, Nowicki MO, Bronisz A, Williams S, Otsuki A, et al. (2008) Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. Cancer Res 68: 9125–9130.
17. Chen Y, Liu W, Chao T, Zhang Y, Yan X, et al. (2008) MicroRNA-21 down-regulates the expression of tumor suppressor PDCD4 in human glioblastoma cell T98G. Cancer Lett 272: 197–205.
18. Huse JT, Brennan C, Hambardzumyan D, Wee B, Pena J, et al. (2009) The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. Genes Dev 23: 1327–1337.
19. Roa W, Brunet B, Guo L, Amanie J, Fairchild A, et al. (2010) Identification of a new microRNA expression profile as a potential cancer screening tool. Clin Invest Med 33: E124.
20. Conti A, Aguennouz M, La Torre D, Tomasello C, Cardali S, et al. (2009) miR-21 and 221 upregulation and miR-181b downregulation in human grade II–IV astrocytic tumors. J Neurooncol 93: 325–332.
21. Lukiw WJ, Cui JG, Li YY, Culicchia F (2009) Up-regulation of micro-RNA-221 (miRNA-221; chr Xp11.3) and caspase-3 accompanies down-regulation of the survivin-1 homolog BIRC1 (NAIP) in glioblastoma multiforme (GBM). J Neurooncol 91: 27–32.
22. Quintavalle C, Garofalo M, Zanca C, Romano G, Iaboni M, et al. (2011) miR-221/222 overexpression in human glioblastoma increases invasiveness by targeting the protein phosphate PTPmu. Oncogene.
23. Skalsky RL, Cullen BR (2011) Reduced expression of brain-enriched microRNAs in glioblastomas permits targeted regulation of a cell death gene. PLoS One 6: e24248.
24. Webster RJ, Giles KM, Price KJ, Zhang PM, Mattick JS, et al. (2009) Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7. J Biol Chem 284: 5731–5741.
25. Kefas B, Godlewski J, Comeau L, Li Y, Abounader R, et al. (2008) microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. Cancer Res 68: 3566–3572.
26. Li WB, Ma MW, Dong LJ, Wang F, Chen LX, et al. (2011) MicroRNA-34a targets notch1 and inhibits cell proliferation in glioblastoma multiforme. Cancer Biol Ther 12: 477–483.
27. Li Y, Guessous F, Zhang Y, Dipierro C, Kefas B, et al. (2009) MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes. Cancer Res 69: 7569–7576.

28. Smits M, Nilsson J, Mir SE, van der Stoop PM, Hulleman E, et al. (2010) miR-101 is down-regulated in glioblastoma resulting in EZH2-induced proliferation, migration, and angiogenesis. Oncotarget 1: 710–720.
29. Xi Y, Nakajima G, Gavin E, Morris CG, Kudo K, et al. (2007) Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. RNA 13: 1668–1674.
30. Weng L, Wu X, Gao H, Mu B, Li X, et al. (2010) MicroRNA profiling of clear cell renal cell carcinoma by whole-genome small RNA deep sequencing of paired frozen and formalin-fixed, paraffin-embedded tissue specimens. J Pathol 222: 41–51.
31. Li J, Smyth P, Flavin R, Cahill S, Denning K, et al. (2007) Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells. BMC Biotechnol 7: 36.
32. Nonn L, Vaishnav A, Gallagher L, Gann PH (2010) mRNA and micro-RNA expression analysis in laser-capture microdissected prostate biopsies: valuable tool for risk assessment and prevention trials. Exp Mol Pathol 88: 45–51.
33. Leite KR, Canavez JM, Reis ST, Tomiyama AH, Piantino CB, et al. (2011) miRNA analysis of prostate cancer by quantitative real time PCR: comparison between formalin-fixed paraffin embedded and fresh-frozen tissue. Urol Oncol 29: 533–537.
34. Wang H, Ach RA, Curry B (2007) Direct and sensitive miRNA profiling from low-input total RNA. RNA 13: 151–159.
35. Hasemeier B, Christgen M, Kreipe H, Lehmann U (2008) Reliable microRNA profiling in routinely processed formalin-fixed paraffin-embedded breast cancer specimens using fluorescence labelled bead technology. BMC Biotechnol 8: 90.
36. Hui AB, Shi W, Boutros PC, Miller N, Pintilie M, et al. (2009) Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. Lab Invest 89: 597–606.
37. Hoefig KP, Thorns C, Roehle A, Kaehler C, Wesche KO, et al. (2008) Unlocking pathology archives for microRNA-profiling. Anticancer Res 28: 119–123.
38. Siebolts U, Varnholt H, Drebber U, Dienes HP, Wickenhauser C, et al. (2009) Tissues from routine pathology archives are suitable for microRNA analyses by quantitative PCR. J Clin Pathol 62: 84–88.
39. Lawrie CH (2007) MicroRNA expression in lymphoma. Expert Opin Biol Ther 7: 1363–1374.
40. Zhang X, Chen J, Radcliffe T, Lebrun DP, Tron VA, et al. (2008) An array-based analysis of microRNA expression comparing matched frozen and formalin-fixed paraffin-embedded human tissue samples. J Mol Diagn 10: 513–519.
41. Glud M, Klausen M, Gniadecki R, Rossing M, Hastrup N, et al. (2009) MicroRNA expression in melanocytic nevi: the usefulness of formalin-fixed, paraffin-embedded material for miRNA microarray profiling. J Invest Dermatol 129: 1219–1224.
42. Nelson PT, Baldwin DA, Scearce LM, Oberholtzer JC, Tobias JW, et al. (2004) Microarray-based, high-throughput gene expression profiling of microRNAs. Nat Methods 1: 155–161.
43. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.

OPEN ACCESS Freely available online

PLOS ONE

Definition of miRNAs Expression Profile in Glioblastoma Samples: The Relevance of Non-Neoplastic Brain Reference

Michela Visani[1], Dario de Biase[1,2]*, Gianluca Marucci[2], Cristian Taccioli[3], Agostino Baruzzi[4], Annalisa Pession[1], the PERNO Study group¶

1 Department of Experimental Pathology, University of Bologna, Bologna, Italy, **2** Department of Hematologic and Oncological Sciences, University of Bologna, Bologna, Italy, **3** Department of Cancer Biology, Paul O'Gorman Cancer Institute, University College London, London, United Kingdom, **4** Department of Biomedical & Neuromotor Sciences, University of Bologna and IRCCS Istituto delle Scienze Neurologiche di Bologna, Bologna, Italy

Abstract

Glioblastoma is the most aggressive brain tumor that may occur in adults. Regardless of the huge improvements in surgery and molecular therapy, the outcome of neoplasia remains poor. MicroRNAs are small molecules involved in several cellular processes, and their expression is altered in the vast majority of tumors. Several studies reported the expression of different miRNAs in glioblastoma, but one of the most critical point in understanding glioblastoma miRNAs profile is the comparison of these studies. In this paper, we focused our attention on the non-neoplastic references used for determining miRNAs expression. The aim of this study was to investigate if using three different non-neoplastic brain references (normal adjacent the tumor, commercial total RNA, and epileptic specimens) could provide discrepant results. The analysis of 19 miRNAs was performed using Real-Time PCR, starting from the set of samples described above and the expression values compared. Moreover, the three different normal RNAs were used to determine the miRNAs profile in 30 glioblastomas. The data showed that different non-neoplastic controls could lead to different results and emphasize the importance of comparing miRNAs profiles obtained using the same experimental condition.

Citation: Visani M, de Biase D, Marucci G, Taccioli C, Baruzzi A, et al. (2013) Definition of miRNAs Expression Profile in Glioblastoma Samples: The Relevance of Non-Neoplastic Brain Reference. PLoS ONE 8(1): e55314. doi:10.1371/journal.pone.0055314

Editor: Daniel Monleon, Instituto de Investigación Sanitaria INCLIVA, Spain

Received August 10, 2012; **Accepted** December 21, 2012; **Published** January 29, 2013

Funding: No current external funding sources for this study. PERNO study group (Region Emilia Romagna, www.perno.it) contributed reagents. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: dario.debiase@unibo.it

¶ Membership of the PERNO Study group is provided in the Acknowledgments.

Introduction

MicroRNAs (miRNAs) are small RNA molecules involved in several cellular processes. Briefly, these small RNAs regulate proteins expression by binding target mRNAs with a perfect or imperfect complementarity [1]. The miRNAs expression analysis could be performed using different techniques, such as microarray assays or Real-Time PCR. Regardless of the chosen approach, one of the most important decisions before analyzing miRNAs profile (as well as for mRNAs expression studies) is the selection of a reference control. The availability of non-neoplastic specimens used as reference is often subordinated to understudied tissue. Differently from what happens for other tissues such as breast or lung [2–4], obtaining brain specimens from healthy subjects is very difficult and, therefore, finding a suitable non-neoplastic control for the analysis of RNA in brain neoplasia still remains a big issue. Moreover, for surgical neoplastic brain samples, the non-neoplastic area is usually absent, very limited, or adjacent the tumor, as for glioblastoma (GBM).

This study was conducted within the PERNO (Progetto Emiliano-Romagnolo di Neuro-Oncologia) project. One of the goals of PERNO is to investigate the role of miRNAs in GBM. In a previous paper [5], we demonstrated the feasibility of miRNAs analysis in brain specimens starting from formalin-fixed and paraffin-embedded tissues (FFPE), as well as in fresh/frozen samples. In literature, there are at least three different specimens used as normal reference for miRNAs analysis in brain samples: the normal area adjacent the tumor [6–8], one of the available commercial references (FirstChoice® Human Brain Reference RNA, Ambion) [9,10], and the tissue removed in epileptic patients [11,12]. Before looking for miRNAs profile in GBM, we decided to deeply investigate the miRNAs expression values in these three different non-neoplastic RNAs.

The aim of this study was to compare three different references used as non-neoplastic control for miRNAs analysis in GBM (the normal area adjacent the tumor, a commercial reference, and the tissue removed in epileptic patients) by investigating the expression levels of nineteen miRNAs. In order to clarify if the choice of non-neoplastic samples could influence the miRNAs analysis in GBM, the miRNAs profiles of thirty GBMs were also investigated using each one of the three references as control.

Materials and Methods

Ethic Statement

The study was approved by Ethic Committee of Azienda Sanitaria Locale di Bologna (number of study 08075, protocol number 139/CE of 5th February 2009, Bologna, Italy). All patients signed a written consent for molecular analysis and anonymous data publication for scientific studies, and all information regarding the human material used in this study was managed using anonymous numerical codes.

Selection of Cases

MicroRNAs expression analysis was performed using a commercial brain reference (FirstChoice® Human Brain Reference RNA, Ambion, Austin, TX, USA), 15 cases of normal samples adjacent the tumor and 15 cases of polar temporal cortical (PTC) specimens removed in patients submitted to surgery (tailored polar anterior temporal resection along with uncus-amygdalohippocampectomy) for drug-resistant epilepsy. All the cases were retrieved at Bellaria Hospital (Section of Pathology, Bologna, Italy), and normal samples adjacent the tumor specimens were included within the PERNO project.

Normal adjacent the tumor. Normal adjacent the tumor tissues were retrieved at a distance between 1 and 2 cm from the margin of 15 primary FFPE GBMs. Patients were 8 males and 7 females, aged 50 to 75 years (mean 62.7 yrs). All samples were diagnosed as GBM according to the 2007 WHO criteria [13]. Thirty samples were also used for the GBMs profile (see below).

Commercial reference. The FirstChoice® Human Brain Reference RNA from Ambion was used. According to the manufacturers' data sheet, it was obtained from several normal brain regions (meaning free of brain pathology) of 23 donors, 13 males and 10 females, aged 23 to 86 (mean 69.7 yrs). FirstChoice® is certified to contain small RNAs, including miRNAs.

Epileptic tissue. Fifteen FFPE PTC samples were randomly selected. Epileptic patients were 7 males and 8 females, aged 25 to 52 years (mean 39.7 yrs). All of them presented drug-resistant anteromedial temporal lobe epilepsy. Histologically, eleven cases showed focal cortical dysplasia while four patients had hippocampal sclerosis. None of them were affected by a neoplastic lesion, including GBM. The tissue used for miRNAs extraction was taken from the temporal lobe cortex.

Glioblastoma. Thirty patients were selected for determining GBMs profile using the three different non-neoplastic references. All specimens were primary GBMs, and patients had not undergone neoadjuvant therapy before surgery. Patients were 14 males and 16 females, aged 42 to 75 years (mean 63.3 yrs). All samples were diagnosed as GBM according to the 2007 WHO criteria [13].

miRNAs Analysis

The hematoxylin and eosin (H&E) sections were reviewed by a pathologist (GM) to select the more informative block. Four 20 μm-thick sections were cut, followed by one H&E control slide. The area selected for the analysis was marked on the control slide to ensure, whenever possible, greater than 90% content of glial cells (normal adjacent the tumor and epileptic specimens) or neoplastic cells (glioblastoma samples).

Nineteen miRNAs (miR-7, miR-9, miR-9*, miR10a, miR10b, miR-17, miR-20a, miR-21, miR-26a, miR-27a, miR-31, miR-34a, miR-101, miR-137, miR-182, miR-221, miR-222, miR-330, miR-519d) were studied according to their role in GBM and because of their previous technical validation in order to determine the feasibility of analysis starting from FFPE tissues [5]. Three small RNAs (RNU49, U54, miR-103) were used as internal control [5]. The miRNAs extraction and analysis were performed as previously described [5]. Briefly, RNA was retrotranscribed using the NCode miRNA First-Strand cDNA Synthesis and qRT-PCR Kits (Invitrogen, Carlsbad, CA, USA), and miRNAs expression was evaluated using an AB7000 machine (Applied Biosystem, Foster City, CA, USA). Each miRNA was run twice per each sample. Considering that commercial reference was a pool of RNA obtained from normal brain, it was analyzed three times (technical replicates).

Statistical Analysis

Expression values and fold changes were obtained by relative quantification and $2^{-\Delta\Delta CT}$ method [14] using the DataAssist 2.0 Tool (Applied Biosystem, Foster City, CA, USA). In order to determine miRNAs profile obtained in GBM, the median fold-change of each miRNA in the 30 GBM samples was compared with "control samples" (15 epileptic specimens, 14 normal adjacent tissues and 1 commercial reference). A GBM/Control ratio <-2.0 means that miRNA was downregulated, while a ratio ≥ 2.0 means that miRNA was upregulated. Statistical analysis of miRNAs expression was performed using GraphPad Prism 5.0 tool. Gaussian distribution was evaluated by Shapiro-Wilk Test. Correlation analysis between miRNAs expression in the three different groups were performed using Spearman correlation test. For comparing the expression levels of each miRNA obtained in the three groups, Kruskal-Wallis and Mann-Whitney tests were used. Level of significance was $p<0.05$ for all the statistical analysis.

Results

All the samples, except one normal adjacent the tumor specimen, gave sufficient quantity of miRNAs for performing the analysis.

miRNAs Analysis in Normal References

Distribution for normal adjacent the tumor, commercial reference, and epileptic groups was not Gaussian as demonstrated by the Shapiro Test ($p<0.001$). For this reason, we used non-parametric statistical tests.

All Spearman correlation values between the expression levels of each miRNAs obtained in the three groups were above 0.65 ($p<0.0001$) (Table 1).

While comparing the median expression values obtained in the three different groups, we observed statistical significant differences ($p<0.05$) in 9 miRNAs: miR-7, miR-9, miR-10a, miR-10b, miR-26a, miR-27a, miR-31, miR-137, and miR-182. For the others, no significant differences were observed (Table S1). Moreover, the Mann-Whitney test, performed considering groups in pairs, revealed statistical significant differences even in miR-101 and miR-519d, as shown in Figure 1. It should be considered that the variability observed in normal adjacent the tumor and in epileptic specimens is a biological variability, while the one observed in commercial reference (a pool of normal brain RNA) is a technical variability.

GBM Profile

The differences observed when comparing expression values of miRNAs in the three different references led us to further investigate if the choice of non-neoplastic control could give discrepant results in analyzing GBM miRNAs profile. For this reason, we compared the profile of the 19 miRNAs in thirty GBMs matched with the three different non-neoplastic brain references

Table 1. Spearman correlation values between three groups ($p<0.0001$).

	Normal Adjacent Tumor	*Ambion Brain Reference*	*Epileptic Tissue*
Normal Adjacent Tumor	/	0.724	0.702
Ambion Brain Reference	0.724	/	0.848
Epileptic Tissue	0.702	0.848	/

doi:10.1371/journal.pone.0055314.t001

(normal adjacent the tumor, commercial reference, and epileptic tissue).

Using different non-neoplastic reference groups resulted in different GBM miRNAs expression profiles (Table 2 and Figure S1). For example, miR-17 was up-regulated (FC $\geq$2.0) in GBM when matched with Ambion reference, but was not deregulated when matched with normal adjacent the tumor or epileptic tissue; miR-31 was down-regulated (FC <-2.0) in GBMs matched with Ambion reference and normal adjacent the tumor, but not deregulated when matched with epileptic tissue. Other miRNAs with different expression status were miR-10a, miR-10b, miR-20a, miR-26a, miR-27a, miR-34a, miR-101, miR-182, miR-221, miR-222, and miR-330, as shown in Table 2.

The remnant miRNAs (miR-7, miR-9, miR-9*, miR-21, miR-137, miR-519d) showed the same expression profile in the three groups even if differences in the level of up- or down-regulation could be observed (Table 2).

Discussion

GBM is the most aggressive brain tumor that may occur in adults. Nevertheless, there were improvements in surgery, radiotherapy, chemotherapy, and "target therapy" [15], while its prognosis remains poor [13,16]. MicroRNAs expression seems to play an important role in cancer development and progression and could be a possible target for molecular therapy [17]. For this reason, identifying a miRNAs profile in GBM could be very useful in developing new drugs and therapeutic approaches. The starting material and samples used as reference control are two crucial points for expression study design. In a previous study, the authors demonstrated that miRNAs analysis in GBM is feasible in FFPE samples, as well as in fresh/frozen ones [5]. Due to the difficulty of gathering non-neoplastic brain specimens, in literature, there are

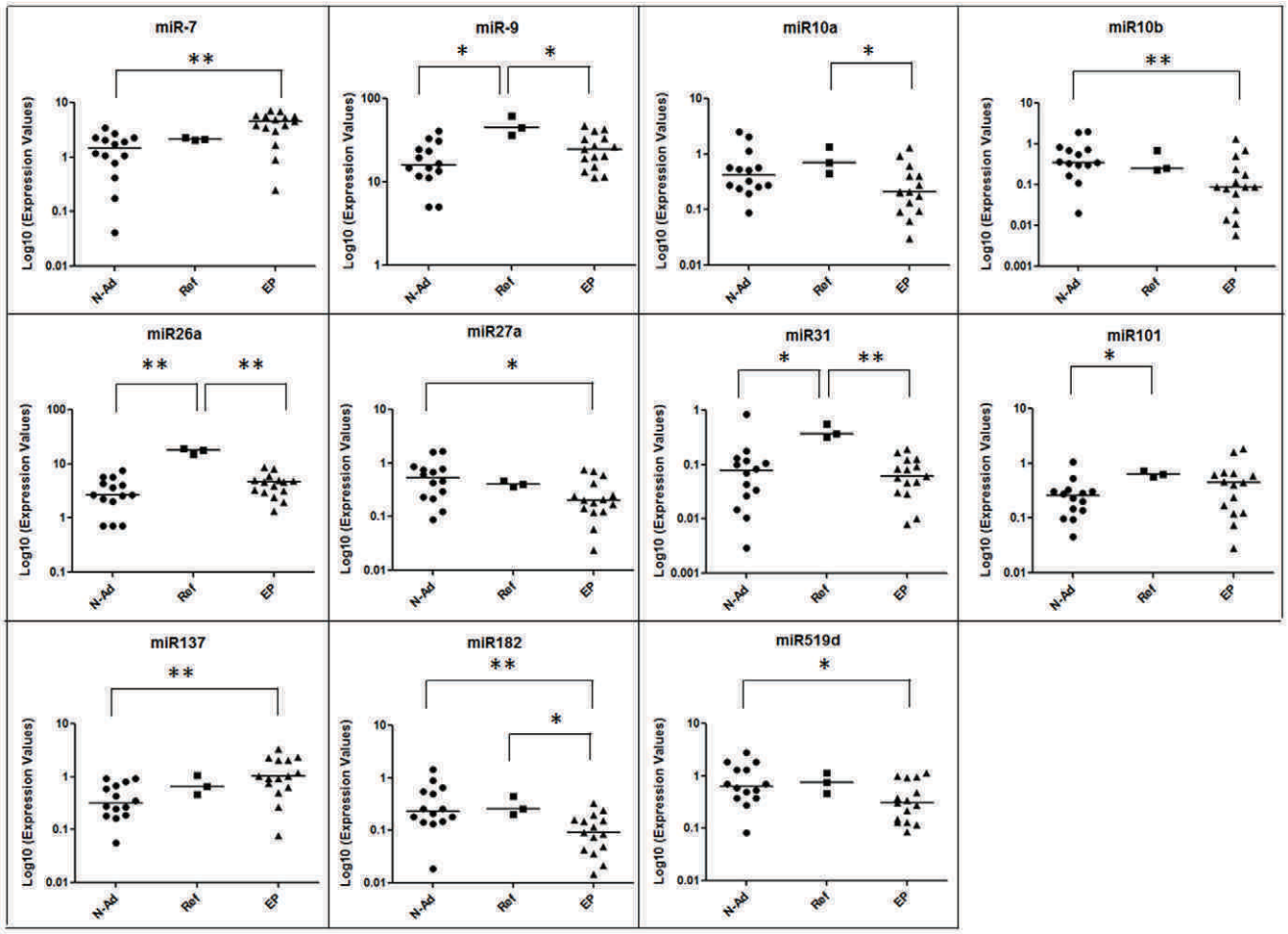


Figure 1. Differences in miRNAs expression. Scatter plots show miRNAs significantly different between groups. Bars indicate median values. * $p<0.05$, ** $p<0.01$ according to Mann-Whitney test. The representation of commercial reference (a pool of normal brain RNA) indicates technical variability, while scatter plots of Normal adjacent the tumor and epileptic specimens show individual variability. N-Ad, Normal adjacent the tumor; Ref, Commercial reference; EP, epileptic.
doi:10.1371/journal.pone.0055314.g001

Table 2. MiRNAs profile in 30 GBMs compared with the 3 different non-neoplastic references.

miRNAs	Normal adjacent Tumor			Ambion Reference			Epileptic		
	Median FC ± Median Error	*Status*[a]	*N. of cases*[b]	*Median FC ± Median Error*	*Status*[a]	*N. of cases*[b]	*Median FC ± Median Error*	*Status*[a]	*N. of cases*[b]
miR-7	−7.377±0.257	DOWN	24/30	−11.086±0.117	DOWN	27/30	−23.753±0.055	DOWN	28/30
miR-9	1.679±0.671	=	14/30	−1.403±0.194	=	18/30	−1.331±0.290	=	20/30
miR-9*	1.855±1.509	=	14/30	1.684±0.936	=	18/30	1.521±0.884	=	18/30
miR-10a	1.296±0.494	=	16/30	1.074±0.280	=	18/30	2.436±0.954	UP	19/30
miR-10b	1.844±1.111	=	9/30	3.105±1.157	UP	21/30	4.688±2.699	UP	23/30
miR-17	1.660±1.620	=	18/30	2.038±1.358	UP	16/30	1.960±1.360	=	14/30
miR-20a	2.396±0.729	UP	18/30	1.606±0.334	=	18/30	2.217±0.501	UP	17/30
miR-21	10.180±3.602	UP	27/30	9.694±2.343	UP	27/30	13.614±5.270	UP	28/30
miR-26a	1.080±1.346	=	21/30	−5.974±0.143	DOWN	28/30	−1.450±0.964	=	19/30
miR-27a	1.419±0.339	=	22/30	2.995±0.489	UP	23/30	2.923±0.764	UP	23/30
miR-31	−3.142±0.775	DOWN	19/30	−8.838±1.471	DOWN	25/30	−1.891±1.041	=	11/30
miR-34a	1.029±0.674	=	15/30	2.205±0.983	UP	15/30	1.928±1.481	=	14/30
miR-101	−1.116±0.675	=	20/30	−2.466±0.209	DOWN	18/30	−2.656±0.241	DOWN	19/30
miR-137	−3.681±0.075	DOWN	24/30	−6.175±0.031	DOWN	29/30	−10.929±0.308	DOWN	29/30
miR-182	−1.049±1.394	=	12/30	1.924±1.923	=	11/30	4.737±1.756	UP	21/30
miR-221	−1.431±0.951	=	15/30	−1.267±0.733	=	14/30	−2.532±0.447	DOWN	18/30
miR-222	−10.230±0.194	DOWN	26/30	−1.982±0.674	=	10/30	−12.987±0.152	DOWN	28/30
miR-330	−4.765±0.228	DOWN	24/30	−1.715±0.432	=	14/30	−5.882±0.156	DOWN	24/30
miR-519d	−4.813±0.238	DOWN	22/30	−3.552±0.220	DOWN	20/30	−2.421±0.326	DOWN	17/30

[a]Status is determined according to Median Fold Change; [b] Number of GBMs showing the modulation out of a total of 5. FC: Fold change; UP: up-regulated (FC ≥2.0); DOWN: down-regulated (FC <−2.0); =: not deregulated.

doi:10.1371/journal.pone.0055314.t002

different samples chosen as reference control in miRNAs expression analysis (e.g. normal adjacent tissues [6–8] or epileptic samples [11,17]). Moreover, several commercial pools of RNAs obtained from normal brain tissues were available, such as the Ambion FirstChoice® Human Brain Reference RNA [6,16]. This situation led each group to arbitrarily choose a reference, sometimes obtaining different miRNAs expression profiles according to selected control [18].

In our study, we investigated if miRNAs expression profiles obtained using different non-neoplastic controls are comparable or not. For this reason, normal samples adjacent the tumor, commercial reference (FirstChoice® Human Brain Reference RNA – Ambion), and epileptic samples were used. Although microarrays are a widescreen and powerful method for miRNAs analysis, we focused on the 19 miRNAs previously analyzed and validated in order to determine the feasibility of analysis starting from both fresh frozen and FFPE tissues [5].

Before analyzing miRNAs expression data, some technical issues regarding the present study should be considered. The mean age of the epileptic group was significantly different from that of the others, as expected considering mean age of epilepsy onset. The commercial reference was a pool of RNAs obtained from multiple donors and several brain regions, while RNAs from other non-neoplastic groups (normal adjacent tissue and epileptic specimens) were not pooled together; for this reason, the replicates obtained from commercial reference represented technical replicates, while those obtained from the other groups were evaluated as biological replicates. Bearing in mind these issues, it should be considered that the aim of the present study was to determine whether GBM miRNAs profile shows differences using several non-neoplastic references. For this reason we reproduced three experimental conditions with normal adjacent tissues, commercial references or epileptic specimens as non-neoplastic controls.

The comparison between expression values of miRNA obtained in each of the three groups revealed good correlation values (>0.65). However, the correlation value was higher when comparing epileptic and commercial reference (R: 0.848). Meanwhile, when epileptic group and commercial reference were compared with normal adjacent the tumor, the correlation values were lower (R: 0.702 and 0.724, respectively). This could be due to the fact that the miRNAs expression profile of normal adjacent the tumor tissue could be influenced by the surrounding neoplastic cells, just as what happened during mRNA expression analysis experiments [19].

While comparing the median expression values of each miRNAs obtained in the three different groups, we observed some statistical significant differences ($p<0.05$) in several miRNAs (miR-7, miR-9, miR-10a, miR-10b, miR-26a, miR-27a, miR-31, miR-101, miR-137, miR-182, miR-519d).

Bearing in mind this evidence, we analyzed 19 miRNAs in a group of GBMs (thirty samples within the PERNO project cohort) using the three previously described references as non-neoplastic controls. We observed that miRNAs profiles obtained in these 30 GBMs were different according to the chosen control group. In fact, no differences were observed in 6 miRNAs, while 13 out of 19 (miR-10a, miR-10b, miR-17, miR-20a, miR-26a, miR-27a, miR-31, miR-34a, miR-101, miR-182, miR-221, miR-222, and miR-330) showed a different modulation in GBM depending on a selected reference, considering a cutoff of 2-fold change. Moreover, it should be noticed that, even in those miRNAs showing a

comparable status in the three groups, differences in fold change values can be observed (e.g. miR-7, miR-137).

The differences in expression of some miRNAs in comparison with other studies could be due to: 1) the enrichment in neoplastic cells could give discrepant results with those obtained without performing dissection [5]; 2) different reference controls could lead to different miRNAs profiles as demonstrated in this study. An example was the study by Malzkorn et al. [10] in which miR-9, miR-17, miR-20a, and miR-21 showed an increased expression in recurrent GBMs compared with primary grade II tumors. Although a splendid approach and technique were used in the study, it is not advisable to compare these results with ours, both in agreement (e.g. mir-20a) and not (e.g. miR-9), because of a different reference (primary grade II tumors) used by Malzkorn et al. for determining the modulation of selected miRNAs.

Even though only 19 miRNAs were here considered, it is reasonable to hypothesize that the same discrepancies could be observed analyzing any miRNAs.

In conclusion, the present study shows that comparing miRNAs profiles obtained using different non-neoplastic controls is not recommended for several reasons: 1) the physiological differences in mean age that could be observed between different groups (e.g. epileptic specimens have a mean age lower than normal adjacent the tumor samples); 2) technical issues: e.g. a commercial reference is usually obtained pooling together several non-neoplastic RNAs (technical variability), while RNAs obtained from normal adjacent the tumour or epileptic specimens are not usually pooled together (biological variability); 3) different selected non-neoplastic groups could have real different miRNAs expression values. Having considered that the number of GBMs analyzed in this study was too small for determining a conclusive miRNAs profile (study in progress), we emphasized that the results of miRNAs profile in GBMs are strictly dependent on the non-neoplastic reference.

Supporting Information

Figure S1 miRNAs profile in 30 GBMs compared with the three different non-neoplastic references. Lines in correspondence of Median FC $=+2$ and -2 indicate the cut off for up- or down-regulation, respectively. Bars indicate FC median errors. FC, Fold change; N-Ad, Normal adjacent the tumor; Ref, Commercial reference; EP, epileptic.
(TIF)

Table S1 Median expression values obtained in the three different groups. *p-values were obtained using Kruskal-Wallis test.
(DOC)

Acknowledgments

The PERNO Study group (Affiliations are indicated between brackets).

Steering committee:

Baruzzi A. (**Chair**), Albani F., Calbucci F., D'Alessandro R., Michelucci R. (IRCCS Institute of Neurological Sciences, Bologna, Italy), Brandes A. (Department of Medical Oncology, Bellaria-Maggiore Hospitals, Bologna, Italy), Eusebi V. (Department of Hematology and Oncological Sciences "L. & A. Seràgnoli," Section of Anatomic Pathology at Bellaria Hospital, Bologna, Italy), Ceruti S., Fainardi E., Tamarozzi R. (Neuroradiology Unit, Department of Neurosciences and Rehabilitation, S. Anna Hospital, Ferrara, Italy), Emiliani E. (Istituto Oncologico Romagnolo, Department of Medical Oncology, Santa Maria delle Croci Hospital, Ravenna, Italy), Cavallo M. (Division of Neurosurgery, Department of Neurosciences and Rehabilitation, S. Anna Hospital, Ferrara, Italy).

Executive committee:

Franceschi E., Tosoni A. (Department of Medical Oncology, Bellaria-Maggiore Hospitals, Bologna, Italy), Cavallo M. (Division of Neurosurgery, Department of Neurosciences and Rehabilitation, S. Anna Hospital, Ferrara, Italy), Fiorica F. (Department of Radiation Oncology, S. Anna Hospital, Ferrara, Italy), Valentini A. (Division of Neurosurgery, Nuovo Ospedale Civile S. Agostino-Estense, Baggiovara, Modena, Italy), Depenni R. (Department of Oncology, Policlinico di Modena, Italy), Mucciarini C. (Department of Oncology, Ramazzini Hospital, Carpi, Modena, Italy), Crisi G. (Department of Neuroradiology, Maggiore Hospital, Parma, Italy), Sasso E. (Department of Neurological Sciences, Maggiore Hospital, Parma, Italy), Biasini C., Cavanna L. (Department of Oncology and Hematology, Guglielmo da Saliceto Hospital, Piacenza, Italy), Guidetti D. (Department of Neurology, Guglielmo da Saliceto Hospital, Piacenza, Italy), Marcello N., Pisanello A. (Department of Neurology, Istituto in tecnologie avanzate e modelli assistenziali in oncologia, IRCCS, S. Maria Nuova Hospital, Reggio Emilia, Italy), Cremonini A.M., Guiducci G. (Division of Neurosurgery, M. Bufalini Hospital, Cesena, Italy).

Registry Coordination Office: de Pasqua S., Testoni S. (IRCCS Institute of Neurological Sciences, Bologna, Italy).

Participants:

Agati R., Ambrosetto G., Bacci A., Baldin E., Baldrati A., Barbieri E., Bartolini S., Bellavista E., Bisulli F., Bonora E., Bunkheila F., Carelli V., Crisci M., Dall'Occa P., Ferro S., Franceschi C., Frezza G., GrassoV., Leonardi M., Morandi L., Mostacci B., Palandri G., Pasini E., Pastore Trossello M., Poggi R., Riguzzi P., Rinaldi R., Rizzi S., Romeo G., Spagnolli F., Tinuper P., Trocino C. (Bologna), Cerasoli S., Dall'Agata M., Faedi M., Frattarelli M., Gentili G., Giovannini A., Iorio P., Pasquini U., Galletti G., Guidi C., Neri W., Patuelli A., Strumia S. (Forlì-Cesena), Casmiro M., Gamboni A., Rasi F. (Faenza, RA), Cruciani G. (Lugo, RA), Cenni P., Dazzi C., Guidi A.R., Zumaglini F. (Ravenna), Amadori A., Pasini G., Pasquinelli M., Pasquini E., Polselli A., Ravasio A., Viti B. (Rimini), Sintini M. (Cattolica, RN), Ariatti A., Bertolini F., Bigliardi G., Carpeggiani P., Cavalleri F., Meletti S., Nichelli P., Pettorelli E., Pinna G., Zunarelli E. (Modena), Artioli F., Bernardini I., Costa M., Greco G., Guerzoni R., Stucchi C. (Carpi, MO), Iaccarino C., Ragazzi M., Rizzi R., Zuccoli G. (Reggio Emilia), Api P., Cartei F., Fallica E., Granieri E., Latini F., Lelli G., Monetti C., Saletti A., Schivalocchi R., Seraceni S., Tola M.R., Urbini B. (Ferrara), Giorgi C., Montanari E. (Fidenza, PR), Cerasti D., Crafa P., Dascola I., Florindo I., Giombelli E., Mazza S., Ramponi V., Servadei F., Silini EM., Torelli P. (Parma), Immovilli P., Morelli N., Vanzo C. (Piacenza), Nobile C. (Padova).

Full affiliations and postal addresses of PERNO participants are available at the study website: www.perno.it.

Author Contributions

Statistical Analysis: CT. Conceived and designed the experiments: DdB MV AP. Performed the experiments: MV DdB. Analyzed the data: DdB MV GM CT AB AP. Contributed reagents/materials/analysis tools: AP AB PERNO Study Group. Wrote the paper: MV DdB AP.

References

1. Ambros V (2004) The functions of animal microRNAs. Nature 431: 350–355.
2. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, et al. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65: 7065–7070.
3. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, et al. (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9: 189–198.
4. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, et al. (2005) MicroRNA expression profiles classify human cancers. Nature 435: 834–838.
5. de Biase D, Visani M, Morandi L, Marucci G, Taccioli C, et al. (2012) miRNAs Expression Analysis in Paired Fresh/Frozen and Dissected Formalin Fixed and Paraffin Embedded Glioblastoma Using Real-Time PCR. PLoS One 7: e35596.
6. Dong H, Siu H, Luo L, Fang X, Jin L, et al. (2010) Investigation gene and microRNA expression in glioblastoma. BMC Genomics 11 Suppl 3: S16.
7. Ciafre SA, Galardi S, Mangiola A, Ferracin M, Liu CG, et al. (2005) Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem Biophys Res Commun 334: 1351–1358.

8. Sasayama T, Nishihara M, Kondoh T, Hosoda K, Kohmura E (2009) MicroRNA-10b is overexpressed in malignant glioma and associated with tumor invasive factors, uPAR and RhoC. Int J Cancer 125: 1407–1413.
9. Guan Y, Mizoguchi M, Yoshimoto K, Hata N, Shono T, et al. (2010) MiRNA-196 is upregulated in glioblastoma but not in anaplastic astrocytoma and has prognostic significance. Clin Cancer Res 16: 4289–4297.
10. Malzkorn B, Wolter M, Liesenberg F, Grzendowski M, Stuhler K, et al. (2010) Identification and functional characterization of microRNAs involved in the malignant progression of gliomas. Brain Pathol 20: 539–550.
11. Wuchty S, Arjona D, Li A, Kotliarov Y, Walling J, et al. (2011) Prediction of Associations between microRNAs and Gene Expression in Glioma Biology. PLoS One 6: e14681.
12. Silber J, Lim DA, Petritsch C, Persson AI, Maunakea AK, et al. (2008) miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. BMC Med 6: 14.
13. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK (2007) WHO. Classification of Tumours of the Central Nervous System. Lyon: IARC editor.
14. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.
15. Brandes AA, Tosoni A, Franceschi E, Sotti G, Frezza G, et al. (2009) Recurrence pattern after temozolomide concomitant with and adjuvant to radiotherapy in newly diagnosed patients with glioblastoma: correlation With MGMT promoter methylation status. J Clin Oncol 27: 1275–1279.
16. Henriksson R, Asklund T, Poulsen HS (2011) Impact of therapy on quality of life, neurocognitive function and their correlates in glioblastoma multiforme: a review. J Neurooncol 104: 639–646.
17. Dalmay T (2008) MicroRNAs and cancer. J Intern Med 263: 366–375.
18. LeBrun DJ, Li M (2011) MicroRNAs in Glioblastoma Multiforme: Profiling Studies and Therapeutic Impacts. Molecular and Cellular Pharmacology 3: 93–105.
19. Ma XJ, Salunga R, Tuggle JT, Gaudet J, Enright E, et al. (2003) Gene expression profiles of human breast cancer progression. Proc Natl Acad Sci U S A 100: 5974–5979.