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

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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. Nevertheless 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]      |

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 Anatomy Pathologica, Bologna, Italy) and Bufalini (Institute of Anatomy Pathologica, 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 (MCaF, 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 for each miRNA analysed.

miRNAs extraction

The “Fresh/Frozen” specimens and cell lines were processed for miRNAs extraction protocol using miVana miRNA isolation kit (Ambion).

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

| miRNA   | Fw Primer Sequence |
|---------|--------------------|
| hsa-mir-7 | TGGAAAGACACTGATTTTGTT |
| hsa-mir-9 | TCTTTGATATCCTCTGTATG |
| hsa-mir-9* | ATAAACATGATAACCGAAAG |
| hsa-mir-10a | ACCCTCTGATGCGAAATTGT |
| hsa-mir-10b | ACCCTCTGAGCAAGATTGT |
| hsa-mir-17 | CAAAGTGTCTACGTCCAG |
| hsa-mir-20a | TAAAGTGCTTAATGTCCAG |
| hsa-mir-21 | TACCCTTATACGATCTGATG |
| hsa-mir-26a | CAAAGTATCCAGGATGAC |
| hsa-mir-27a | TTCAAGATGCTTAAATGTCC |
| hsa-mir-31 | AGGCAATGATCGCCATA |
| hsa-mir-34a | TGCCAGGTCCTTACGGT |
| hsa-mir-101 | TACAGTATCTGATAAACCAG |
| hsa-mir-137 | TTATTCTCAGAATGACTG |
| hsa-mir-182 | TGTGGCAATGATGAACTCAC |
| hsa-mir-221 | GCTAATCTGCTTCCTGAT |
| hsa-mir-222 | GCTAATCTGCTTCCTGAT |
| hsa-mir-330 | TCTCTGGGCTTGCTTATA |
| hsa-mir-519d | AAGTGCCCTCCTTTAGAGT |

LNA bases are underlined. Fw: forward. Hsa: Homo sapiens (human).
kit (Applied Biosystem, Austin, TX, U.S.A.). Briefly, small RNA fraction was extracted 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 retrotranscription 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://mirorna.sanger.ac.uk) (Table 2). Primers were modified with LNA (Locked

![Figure 1. Scatter plot showing Spearman correlation between Fresh/Frozen and FFPE-dissected groups.](http://example.com/fig1.png)

Figure 1. Scatter plot showing Spearman correlation between Fresh/Frozen and FFPE-dissected groups.
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![Figure 2. Median fold-change calculated per each miRNA between 30 paired Fresh/Frozen and FFPE samples.](http://example.com/fig2.png)

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.
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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 Biosysterm, 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.](https://journals.plos.org/plosone/article/fetchObject.action?uri=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.

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

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