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miR-15b and miR-21 as Circulating Biomarkers for Diagnosis of Glioma

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Abstract: Malignant gliomas are lethal primary intracranial tumors. To date, little information on the role of deregulated genes in gliomas have been identified. As the involvement of miRNAs in the carcinogenesis is well known, we carried out a pilot study to identify, as potential biomarkers, differentially expressed microRNAs in blood samples of patients affected by glioma. We studied the miRNAs' expression, by means of microarray and Real-Time PCR, in 30 blood samples from glioma patients and in 82 blood samples of patients suffering from: (a) various neurological disorders (n=30), (b) primary B-lymphoma of the Central Nervous System (PCNSL, n=36) and (c) secondary brain metastases (n=16). By quantitative real time reverse-transcriptase polymerase chain reaction (qRT-PCR), we identified significantly increased levels of two candidate biomarkers, miR-15b and miR-21, in blood of patients affected by gliomas. ROC analysis of miR-15b biomarker levels allowed to differentiate patients with tumour from patients without glioma. Furthermore, combined expression analyses of miR15b and miR-21 distinguished between patients with and without glioma (90% sensitivity and 100% specificity). In addition, a decrement in the expression levels of miR-16 characterized glioblastomas compared to low grade and anaplastic gliomas. In conclusion, this pilot study suggest that it's possible to identify the disease state by meaning miR-15b and miR-21 markers in blood, while miR-16 can be used to distinguish glioblastoma from other grade gliomas. They can potentially be used as biomarkers for non-invasive diagnosis of gliomas; further studies are mandatory to confirm our preliminary findings.

Keywords: Biomarkers, Blood, Diagnosis, Glioma, Microarrays, miRNAs.

INTRODUCTION

Glioma is the most common primary Central Nervous System (CNS) tumour, including about 50% of primary brain cancers in adults. They originate from the neuroepithelial glial cells and the main malignancy of these tumors is linked to their diffusely infiltrative growth pattern, strong angiogenesis and an intrinsic resistance to chemotherapy and radiotherapy. These aspects make gliomas extremely difficult to treat. Although new therapeutic care and supportive strategies, the median survival of glioblastoma multiforme has not significantly changed over the past decade, being remaining limited to 12–15 months [1]. Even if with the current neuroradiological imaging a high degree of detection due to suspected presence can be reached, the gold standard for the diagnosis remains the histology. miRNAs are small noncoding RNA that can contribute to tumour pathogenesis and progression, as suggested by their different levels of expression in normal tissues and cancers [2-4]. A large portion of overexpressed miRNAs was identified in many human solid tumours, such as colon, breast, prostate, lung, stomach and pancreatic cancers, but also in many circulating body fluids. In blood, circulating miRNAs are abundant, very stable and a fraction of circulating miRNAs, due to encapsulation in an envelop made by proteins and/or lipids, is resistant to plasma RNAses. Furthermore, miRNAs detected in circulating body fluids are relatively stable, highly accessible, not invasive and these are important prerequisites for reliable clinical biomarkers [5, 6]. Circulating miRNAs in the blood of tumour patients can be released during tissue injury or by active delivery and could play the same important role as miRNAs in tissues. However, the mechanisms of delivery and biological function of extracellular miRNAs remain unknown [7]. The microRNA content of cancer cell-derived exosomes is correlated to the microRNA level in the primary tumor in ovarian and lung cancer. miRNAs have been identified in exosomes and microvesicles derived from several tissues [6], such as human and mouse mast cells [8], glioblastoma tumors [9], plasma [10], saliva [11] and urine [12]. For example, miR-150 biomarker, involved in the expression of c-Myb gene, is secreted in microvesicles from human blood cells or cultured THP-1 cells and taken up by HMEC-1 microvascular endothelial cells [13]. It has been demonstrated with several techniques that different tumour types have distinct intracellular miRNA profiles [14-18] and specific expression profiles can distinguish different clinical pathways, as for primary from secondary glioblastoma types [19]. In some cases, the detection of circulating miRNAs has been linked to occasional lysis of blood cells during sample

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preparation [2]. To date, literature data report few circulating miRNAs useful for the detection and risk stratification of gliomas, as serum miR-128, whose expression decreased in glioma preoperative serum compared with normal controls and meningioma serum samples; furthermore, low miR-128 levels in serum and tissue were significantly correlated with high pathological grade [20]. In order to early detect systemic cancer, to predict tumor progression or to check the response to therapy, it was evaluated using of cancer-derived circulating DNA [21-24], as miRNAs in cerebrospinal fluid samples from patients with glioma [25]. The involvement of miRNAs in the carcinogenesis is well known and the possibility to detect their levels in blood samples is appealing for early diagnosis and for monitoring patients. For that reason, we carried out a pilot study with the aim of identify in blood samples of patients affected by glioma differentially expressed microRNAs as potential novel glioma biomarkers.

PATIENTS AND METHODS

Serum Samples and Clinical Information

In this study, a total of 112 plasma samples were analyzed, 30 plasma samples of patients with glioma, 30 from patients with various neurological pathologies, 36 from patients with PCNSL and 16 from individuals with brain metastases or leptomeningeal secondary involvement originated from various tumours. RNA was extracted from sera prepared by centrifugation starting from 10 ml of whole blood, obtained at the time of surgical treatment. All whole blood and serum samples were used with the consent of the patients.

RNA Extraction, Reverse-Transcription and qRT-PCR

Total RNA was extracted using TRIZOL™ reagent (Invitrogen) and microRNA was isolated using a PureLink™ miRNA Isolation Kit (Invitrogen), following manufacturer's instructions. The RNA concentration was determined by NanoDrop ND-3300 spectrophotometer and TaqMan miRNA assays (Applied Biosystems) quantified miRNA levels [26]. Briefly, 10 µL cointaining total RNA was used in reverse-transcription reactions (16°C for 30 min, 42°C for 30 min, 85°C for 5 min, followed by 4°C). Real-time PCR was carried out using a 7500 Real-Time PCR System, with following cycling conditions: 95°C for 10 min, 40 cycles of 15 s at 95°C and 60 s at 60°C. The 7500 SDS system software (version 1.2.3; Applied Biosystems) was used to transform fluorescent data of each sample, run in duplicate, into cycle threshold (Ct) measurements. Mean Ct values and standard deviations were calculated for total miRNAs and the amount of target miRNA was normalized respect to the amount of miR-24, selected from several control miRNAs, as following: $\Delta Ct = \Delta CtmiR - \Delta CtmiR\text{-}24$. Relative Expression Levels (REL) were reported as $2^{-\Delta}$ Ct.

Microarray Fabrication, miRNA Extraction, Labelling and Hybridisation

The epoxy microscope glass slides (Sigma), activated with glycidyloxipropyltrimethoxysilane (GOPTS), immobilise amino-modified oligonucleotide DNA. A 340 custom oligo-array was built with DNA probe complementary to a corresponding full length of 340 mature miRNAs and comprised positive and negative control probes. More informations about microarray protocols can be found at the Gene Expression Omnibus (GEO) at the National Centre for Biotechnology Information (NCBI). Total RNA was extracted from blood/sera using TRIZOL™ reagent (Invitrogen) and miRNA was isolated using a PureLink™ miRNA Isolation Kit (Invitrogen). microRNAs were tagged and hybridized by NCode™ miRNA Labeling System (Invitrogen), according to manufacturer's instructions, and then placed on the microarray slides; each miRNA sample was tagged with Alexa Fluor® 5, while the universal reference RNA was labelled using Alexa Fluor® 3. Each array was subsequently washed and analyzed using an Affymetrix 428 array Scanner.

Statistics

The statistical analysis was performed using SPSS (version 19; SPSS) and GraphPad Prism (version 5.0, GraphPad Software). The 2-tailed Mann-Whitney U tests and Kruskal-Wallis tests with Dunn's multiple comparison were applied for groupwise comparisons of clinical and biological data's distributions. Results were statistically significative for $P<0.05$.

RESULTS

Patients

In this study, we analyzed 112 plasma samples derived from 53 male and 59 female patients; the age was 18–75 years (mean 51 years). In all patients with glial tumour, the histopathologic diagnosis was established by brain biopsy. There were 6 patients with anaplastic astrocytoma (WHO grade III), 8 patients with low-grade astrocytoma (World Health Organization [WHO] grade II) and 16 patients with glioblastoma (WHO grade IV). Primary treating physicians by means of a questionnaire gave preliminary informations. The median interval between cancer and serum sampling was 1 month for both astrocytic and oligodendroglial cancers, but the wide range considers the presence of patients with long follow-up times. In 4 patients in the astrocytic group, blood samples were obtained more than one year after surgery and in 5 patients with oligodendroglial cancers, more than two years after tissue sampling. All serum samples were achieved after surgery and prior to radiotherapy in 60% of the patients. Eighteen of 30 serum samples derived from patients affected by glioma were analyzed by microarray, while all the samples were analyzed by qRT-PCR. Similarly, 18 of the 30 serum samples derived from patients affected by various neurological disorders (control group) were studied by microarray, while all the samples were analyzed by means of qRT-PCR.

The microarray gene expression profile was performed comparing the serum derived from 18 patients affected by glioma against the serum of 18 patients with various neurological disorders in order to find differentially expressed microRNA.

In order to validate the microarray expression profile data we have studied the expression levels of the four differentially expressed microRNAs in a larger cohort of patients composed by 30 patients affected by glioma (18 of which

were already analyzed by microarray), 30 control patients affected by vairous neurological disorders (18 of which were already analyzed by microarray), 30 patients affected by PCNSL and 16 patients affected by metastases.

Differential Expression of miRNAs in Glioma - Gene Expression Profiling

The expression profile of 340 mammalian microRNAs was monitored by DNA microarrays using plasma samples from 18 patients with glioma and plasma from 18 patients suffering from various neurological disorders ("control" group). To avoid low expression genes, only microRNAs with a *Signal* value greater than 100 were selected and miRNAs with a signal to noise ratio greater than 2.5 were chosen for further studies. Afterwards, microRNAs showing different level of expression between glioma and "control" serum were independently filtered by a parametric Student's t test, assuming equal variances, p-value cut-off of 0.01 and multiple testing corrections (Benjamini and Hochberg False Discovery Rate). Using these restrictions, we selected four microRNAs which clearly present a differential expression between glioma and control group: miR-16, miR-15b miR-21 and miR-155. Using Smooth correlation coefficients, all samples were analized by average linkage clustering, on the basis of similarity of expression patterns over the detected genes. This yielded two major clusters, one performing the glioma group and the other the control group, as expected (Fig. **1**).

Diagnostic Analysis of miR-15b and miR-21 Levels in Plasma by qRT-PCR

The four microarray-selected miRNAs were quantified by TaqMan qRT-PCR in a set of serum samples derived from 30 patients affected by glioma (18 of which are the same serum samples used in for microarray gene expression profile) and from 30 "control" patients affected by miscellaneous neurologic disorders (18 of which are the same serum samples used in for microarray gene expression profile).

As expected miR-15b and miR-21 were significantly increased (early Ct values) in serum samples derived from patients affected by glioma. Conversely, miR-16 showed significantly decreased levels in patients with glioma; low levels of miR-24 were measured in all samples (Table **1**). As other classes of small RNAs, such as the snoRNA RNU6B, are not useful for the normalization as they are unstable in serum, it's important to find small housekeeping microRNAs. In this study, miR-24 was proposed for normalisation and showed to be applicable to the analysis of these types of tumors. MiRNA expression levels in individual plasma specimens were therefore reported as RELs. A remarkably increased mean REL miR-15b and a decreased expression of miR-16 was demonstrated in plasma samples from patients with glioma (Fig. **2**).

By ROC curves of miR-15b, we observed evident separations between the glioma patients group and those without tumour, with an area under the curve (AUC) of 1 (for a review on ROC and AUC plots see Zweig *et al.* 1993) [27]. Corresponding to this analysis, a cut off plasma REL with the highest accuracy for miR-15b was determined to be 4 with 100% sensitivity and specificity (Fig. **2**). By combined REL of miR-15b with REL of miR-21 and by a diagnostic tree, we increased the specificity of discrimination of glioma from other diseases (Fig. **3**). For miR-21, a REL of 4 was used to distinguish glioma from PCNSL and brain metastases while a REL of 1,74 was selected to distinguish glioma from "control" samples.

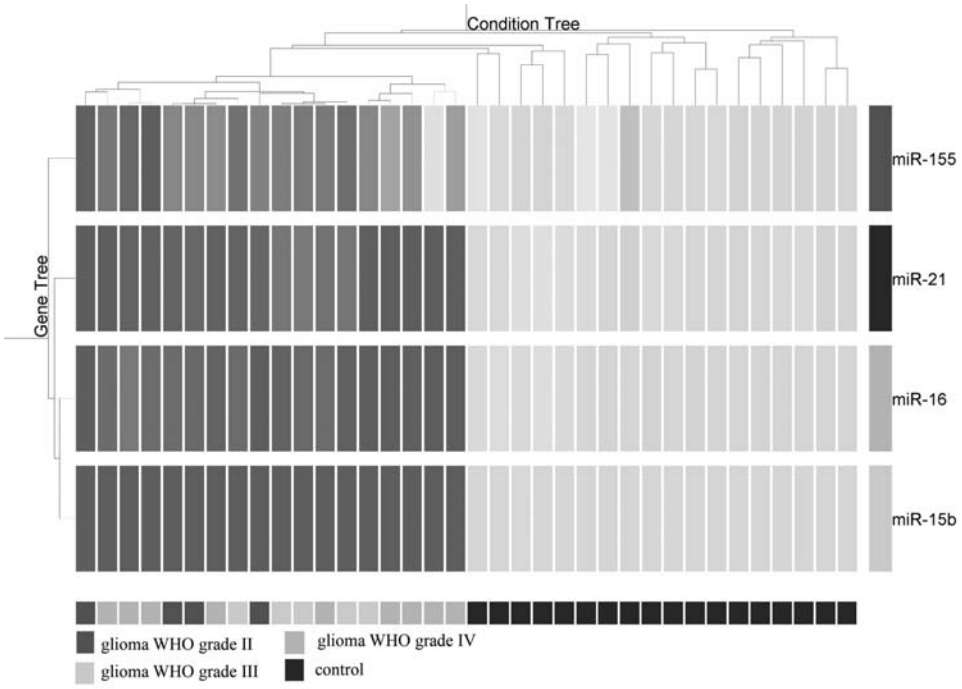

Fig. (1). *Cluster analysis.* Tree generated by a cluster analysis performed on 36 blood samples. MiRNAs expression profile shows a clear separation of 18 patients with glioma from 18 patients suffering various neurological disorders.

Table 1. miRNA expression in blood samples from patients with glioma, compared with control patients.

	Patients with Glioma[a]		Control Patients[b]		p value[e]
	Ct[c]	SD[d]	Ct[c]	SD[d]	
miR-15b	31,79378	0,960109	34,7718	0,84934	<0,01
miR-16	28,58248	1,168615	27,55171	0,550783	0,02
miR-21	31,30626	0,989405	32,4941	0,550783	<0,01
miR-155	34,03455	0,5547	34,00748	0,554491	0,03
miR-24	29,46907	0,280595	29,49652	0,290466	0,01

[a] n=30

[b] Patients with miscellaneous neurological disorder

[c] Data are means of CT values

[d] Standard deviation

[e] The P value is for comparison of miRNA expression among patients with glioma and control patients and was calculated using the Mann-Whitney U test

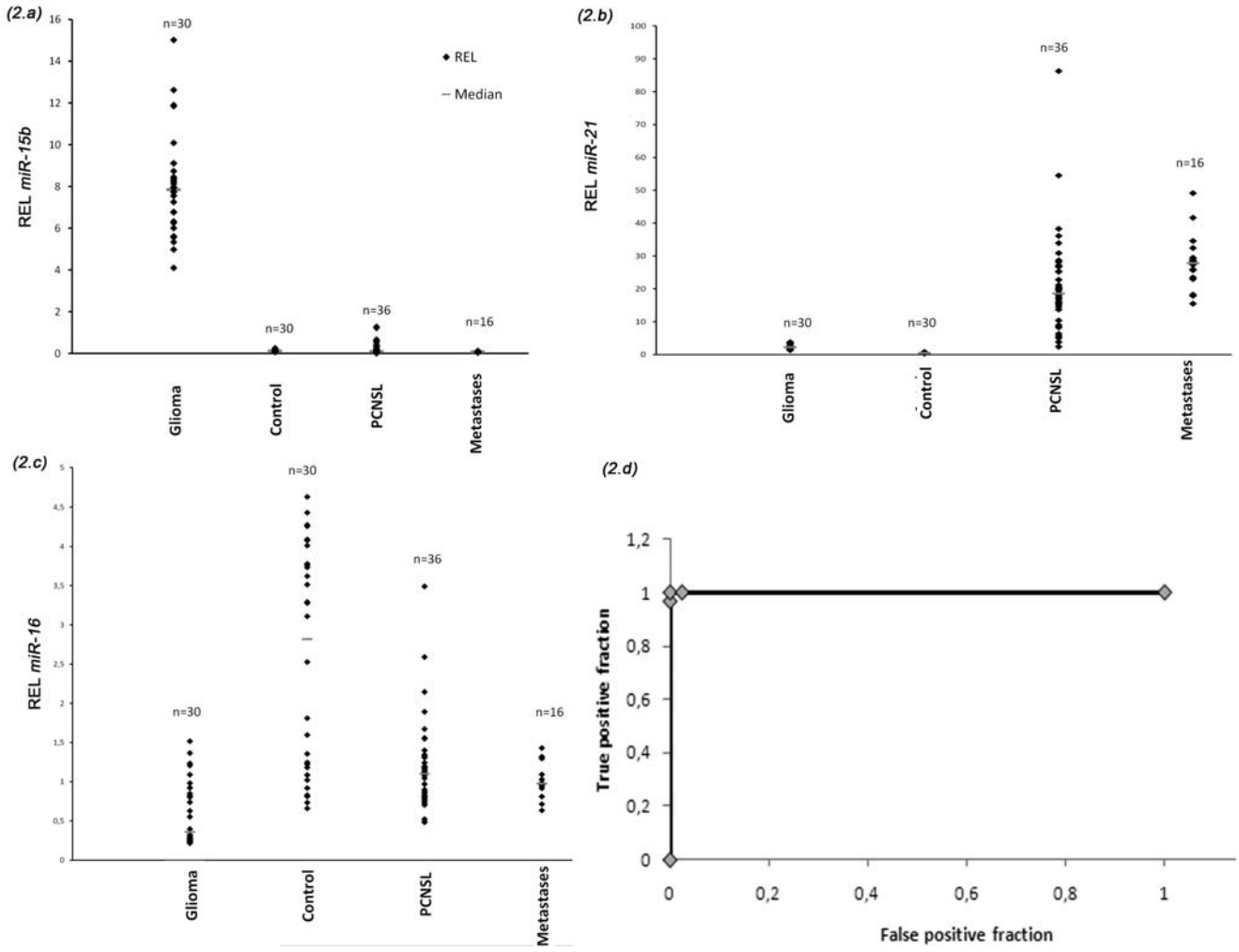

Fig. (2). *Relative expression levels of miR-15b, miR-16 and miR-21 in blood samples from patients with glioma, PCNSL and control patients.* Scatter plots of expression levels of miR-15b (2.a), miR-21 (2.b) and miR-16 (2.c) in blood samples from patients with glioma, miscellaneous CNS disorders, PCNSL and brain or leptomeningeal metastases. Relative Expression Levels (REL) was normalized to expression levels of biomarker miR24 (y-axis); median REL values were represent by black horizontal lines. (2.d) The area under the curve (AUC) of 1 were yielded by blood relative expression of miR-15b as a single biomarker.

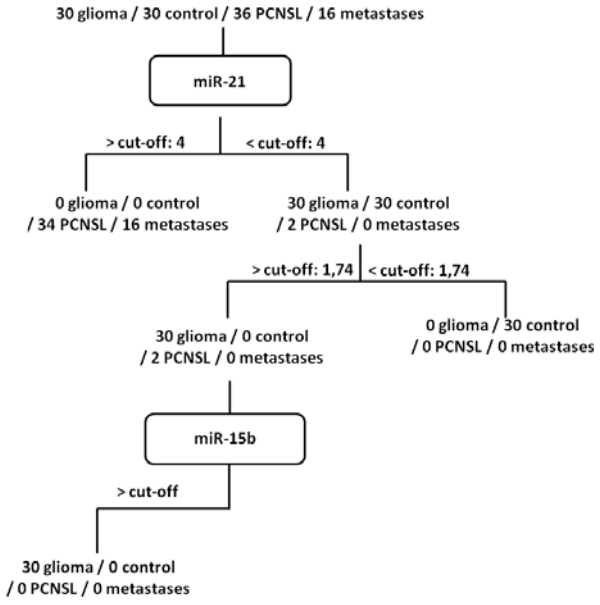

Fig. (3). ***miRNA diagnostic tree*** **-** REL values lower than 4 for miR-21 selected a first subgroup made of 30 gliomas together with 30 control and 2 PCNSL patients. Internal to this group, using another REL range for miR-21 (values between 1.74 and 4) a group made of all gliomas together with 2 PCNSL patients is selected. A further classification is shown using REL values for miR-15b higher than 4, in order to finally select the 30 glioma only.

Between the three miRNAs identified miR-16 only was able to discriminate between the different grades of gliomas, as its expression in the plasma were decreased significantly in glioblastoma (WHO IV) (Fig. **4**). By ROC curves of miR-16, an evident separation was observed between glioblastoma (WHO IV) group and those of other grades (WHO II and III), with an AUC of 0.98. In this analysis, a cut off plasma REL with highest accuracy for miR-16 was 0.33 with 0.98% sensitivity and 98% specificity (Fig. **4**). The only false-negative results were observed for two patients with anaplastic astrocytoma diagnosis (WHO grade III).

DISCUSSION

In the present study, we demonstrated that in patients affected by glioma an altered extracellular production/excretion of miR-15b, miR-16, miR-21 and miR-155 can be detected in peripheral blood samples, providing evidence that microRNAs circulating in plasma may be used as biomarkers for the detection and grading of gliomas. These 4 deregulated miRNAs were quantified by qRT-PCR. In plasma samples collected from subjects with glioma, miR-15b levels were significantly increased compared with patients affected by miscellaneous neurologic disorders, PCNSL or brain metastases and AUC had value 1 in ROC analyses; these results are in agreement with those reported by several other authors, who reported high miR-15b expression levels in the cerebrospinal fluid (CSF) derived from patients with glioma and in glioma tissues [25, 28, 29]. MiR-15b, involved in tumour carcinogenesis by regulating cell cycle progression, has a significant diagnostic value for glioma. Interestingly the biomarker miR-15b, inducing cell cycle arrest in G1 phase by targeting Cyclin E1 (CCNE1) and regulating the cell cycle by G1 to S phase transition, may function as a tumor suppressor, as suggested by Xia *et al.* [28]. By microRNA microarray, miRNA expression levels compared between normal and glioma tissues in Chinese patients were significantly deregulated in glioma samples, as observed for miR-15b, -34a, -146b and -200a; miR-15b was down-regulated also in chronic lymphocytic leukemia and in gastric tumour, modulating multidrug resistance by targeting BCL2 [29, 30].

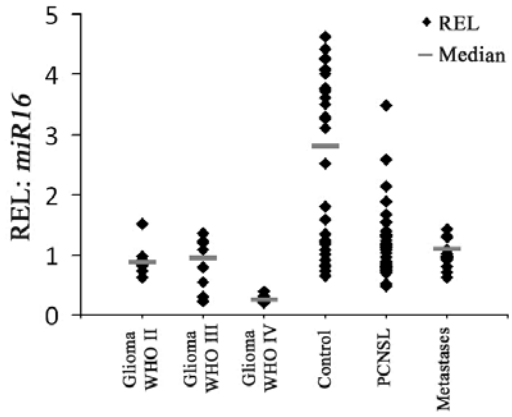

Fig. (4). Relative expression levels of miR-16 in blood samples from patients with glioma WHO II, glioma WHO III, glioma WHO IV, control patients, patients with PCNSL and patients with metastases. Scatter plots of expression levels of miR16 in blood samples collected from patients with various grade glioma, miscellaneous CNS disorders, PCNSL and brain or leptomeningeal metastases. Relative Expression Levels (REL) was normalized to the expression levels of biomarker miR24 (y-axis); median REL values were represent by black horizontal lines.

To increase the discriminatory diagnostic value, we also combined miR-15b and miR-21 expression analyses. Thus, combined analysis of these biomarkers demonstrated that miRNA blood levels accurately distinguished patients with glioma, PCNSL and brain metastases or leptomeningeal carcinoma. For miR-15b, a cut off plasma REL with the highest accuracy was 4, instead for miR-21 a REL of 4 was used to distinguish glioma from PCNSL and brain metastases while a REL of 1.74 was selected to distinguish glioma from "control" samples. More precisely, the REL of these two miRNAs have proved to be of great importance since they have allowed to discriminate between patients with glioma and all the other (specifically, REL values lower than 4 for miR-21 selected all 30 gliomas together with 30 control and 2 PCNSL). Internal to this group, has been possible to use another REL values for miR-21 (values between 1.74 and 4) to select again a group made of all gliomas together with 2 PCNSL patients. An additional classification has been done using REL values for miR-15b higher than 4, to finally select the 30 glioma only (Fig. **3**). MiR-15b, the most abundant microRNA in blood samples, showed an 8-fold higher expression levels in patients with glioma. MiR-21, which was also overexpressed in the cerebrospinal fluid samples from patients with glioma, is one of the most consistently expressed microRNA in cancer originating from other tissues. It functions as an oncogene in glioma, preventing apoptosis.

Several pathways are predicted targets for miR-21, such as the tumor suppressor gene p53 pathway, the transforming growth factor and the mitochondrial apoptosis pathways. Furthermore, studies have shown that tropomyosin 1 could be down-regulated by miR-21. Other predicted targets include programmed cell death gene 4 (PDCD4), the phosphatase and tensin homolog tumor suppressor and reversion-inducing cysteine-rich precursor with Kazal motifs [31, 34]. Noteworthy, glioblastomas expressed significantly higher levels of miR-15b and miR-21 than anaplastic astrocytomas, while there was no significant association between expression levels of these markers and survival among patients with glioma [29]. Consistently with Baraniskin [25], we observed no association between miR-15b and miR-21 expression levels and the survival among patients with tumour or the glioma grading. On the other hand, a correlation was found between glioma grading and the expression levels of miR-16. A decreased expression levels of biomarker miR-16 characterize the glioblastoma (WHO IV) in respect to low grade and anaplastic glioma. These data are in accordance with that of Baraniskin *et al.*, who demonstrated that microRNAs circulating in the cerebrospinal fluid (CSF) can be used as biomarkers for glioma detection [26]. MiR-16, that forms a cluster with miR-15a at chromosome position 13q14, functions by targeting many oncogenes, as BCL2, MCL1, CCND1, WNT3A and these miRNAs are down-regulated in chronic lymphocytic lymphoma (CLL), prostate carcinoma and pituitary adenomas [32, 33].

Cancer-secreted microRNAs are important intermediaries in cancer-host crosstalk and they are investigated for their potential use as prognostic and predictive biomarkers. miR-21 and miR15b may represent an therapeutic target to control multiple steps of pathogenesis; for example, the inhibition of miR-21 in glioblastoma cells increased apoptosis, while in cultured hepatocellular carcinoma cells significantly led to decrease of tumor cell proliferation, migration and invasion [31]. After transfection with anti-miR-21, reduced cellular invasion were observed in a colorectal, breast and prostate cancers cells; moreover, when anti-miR-21 was transfected in metastatic breast cancer cells, the number of lung metastases was significantly decreased [31]. The decrease of the expression levels of miR-15b and miR-200b underlines the mechanisms of epithelial-mesenchymal transition induced by chemotherapy and it might serve as therapeutic targets to reverse chemotherapy resistance in tongue tumours. Low levels of miR-200b and miR-15b were expressed in patients with lymph node metastasis or chemotherapeutic resistance, indicating that tumor progression was associated with miR-200b and miR-15b downregulated levels [35]. Also other microRNAs are investigated for their potential prognostic use. In the breast tumour, miR-105 characteristically was expressed and secreted by metastatic breast cancer cells, as it was a potent regulator of migration targeting the tight junction protein ZO-1. The overexpression of miR-105 in non-metastatic cancer cells induced metastasis and vascular permeability in distant organs, whereas these effects were alleviated in highly metastatic tumors when miR-105 was inhibited [36]. miR-139-5p is identified as a prognostic marker for the aggressive forms of breast tumour; bioinformatic analysis reveales a predicted disruption to the TGFβ, Wnt, Rho and MAPK/PI3K signaling cascades, implying a potential regulated role in the cellular invasion and migration [37]. Metastatic endothelial recruitment, angiogenesis and colonization were suppressed by miR-126, through coordinate targeting of pro-angiogenic genes. Extracellular (exosome-like) vesicles are involved in cell-to-cell communication; breast cancer cells released membrane vesicles into extracellular medium containing potential oncogenic molecules, such as proteins and miRNAs, that could transmit signals to non-malignant cells and implicate tumor progression and metastasis [38].

MiRNA take part in a wide range of biological processes, as immune response, cellular proliferation and apoptosis. Several works evaluate host-pathogen interaction, focused on viral and bacterial infections. Virus express many microRNAs in infected cells to modulate the levels of both viral and cellular mRNAs, thereby influencing viral replication and pathogenesis; less is known about the bacterial infections and the effect of bacterial pathogens on host miRNA expression. In the plant infection, for example, up-regulation of miR-393a induced by Pseudomonas syringae in Arabidopsis thaliana contributes to the resistance of the plant against the bacterial pathogen; as bacterial defense mechanism, conversely, *P. syringae* secretes into the host cell effector proteins that suppress transcription or activity of host microRNAs [39].

In the mammalian infection, the analysis of circulating miRNAs in the serum of individuals infected with Mycobacterium tuberculosis identified 59 up-regulated miRNAs and 33 down-regulated. It has been shown that T-cells can transfer miRNAs to antigen-presenting cells via exosomes, suggesting that intercellular miRNA transfer may contribute to coordinate and fine-tune gene expression during the immune response [39]. Human monocytes stimulated with bacterial wall component lipopolysaccharide (LPS), furthermore, up-regulated miR-132, miR-146a/b and miR-155 targeting mRNAs of genes downstream of Toll-like receptor 4 (TLR4), to protect host cells from an excessive TLR4 response [40, 41]. In the gut mucosa homeostasis, comparative analysis of miRNA expression of germ-free mice and mice colonized with the microbiota from pathogen-free mice identified 9 miRNAs differentially expressed: miR-298 (ileum) and miR-128, miR-200c, miR-665, microR-465c-5p, miR-342-5p, miR-466d-3p, miR-466d- 5p and miR-68 (colon). Dulmasso *et al.* concluded that host microRNA expression is modulated by microbiota and it in turn regulates host gene expression [42].

This study is based on a limited number of miRNAs, considering that the total number of known microRNAs has triplicated in recent years; thus, further studies are required to better understand which are the target of these miRNAs and in which molecular pathways are involved.

CONCLUSION

In this work, considering the high diagnostic value of combined miR-15b, miR-21 and miR-16 analyses, we present a set of circulating miRNA that characterize glioma. In accordance with Baraniskin study in CSF samples, the availability of these miRNA may facilitate the diagnosis and clinical management of this tumour type. As cancer-secreted microRNAs are emerging mediators of cancer-host crosstalk,

they may be used as indicators of early disease detection and recurrence prediction after surgery. These results provide an evident rationale for quantification in prospective trials of microRNAs in plasma for diagnostic and prognostic purposes in brain tumors.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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Declared none.

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