

Identification of microRNAs in the cerebrospinal fluid as biomarker for the diagnosis of glioma

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Malignant gliomas are the most common and lethal primary intracranial tumors. To date, no reliable biomarkers for the detection and risk stratification of gliomas have been identified. Recently, we demonstrated significant levels of microRNAs (miRNAs) to be present in cerebrospinal fluid (CSF) samples from patients with primary CNS lymphoma. Because of the involvement of miRNA in carcinogenesis, miRNAs in CSF may serve as unique biomarkers for minimally invasive diagnosis of glioma. The objective of this pilot study was to identify differentially expressed microRNAs in CSF samples from patients with glioma as potential novel glioma biomarkers. With use of a candidate approach of miRNA quantification by reverse-transcriptase polymerase chain reaction (qRT-PCR), miRNAs with significant levels in CSF samples from patients with gliomas were identified. *MiR-15b* and *miR-21* were differentially expressed in CSF samples from patients with gliomas, compared to control subjects with various neurologic disorders, including patients with primary CNS lymphoma and carcinomatous brain metastases. Receiver-operating characteristic analysis of *miR-15b* level revealed an area under the curve of 0.96 in discriminating patients with glioma from patients without glioma. Moreover, inclusion of *miR-15b* and *miR-21* in combined expression analyses resulted in an increased diagnostic accuracy with 90% sensitivity and 100% specificity to distinguish patients with glioma from control subjects and patients with primary CNS lymphoma. In conclusion, the results of this pilot study demonstrate that *miR-15b* and *miR-21* are markers for

gliomas, which can be assessed in the CSF by means of qRT-PCR. Accordingly, miRNAs in the CSF have the potential to serve as novel biomarkers for the detection of gliomas.

Keywords: cerebrospinal fluid (CSF), glioma, microRNA (miRNA), primary central nervous system lymphoma.

Gliomas represent the most common type of primary CNS cancers, comprising about 50% of primary brain tumors in adults.¹ They arise from glial cells, the neuroepithelial supporting cells of the CNS. Because malignant gliomas show a diffusely infiltrative growth pattern, robust angiogenesis, and an intrinsic resistance to chemotherapy and radiotherapy, these tumors are extremely difficult to treat. Despite implementation of intensive therapeutic strategies and supportive care, the median survival of glioblastoma multiforme has remained at only 12–15 months over the past decade.¹

Histopathology of tumor specimens gained by microsurgical resection or by stereotactic biopsy is the standard diagnostic procedure for patients with glioma-suspected lesions. Neuroimaging, in particular MRI, is instrumental for disease staging and for follow-up. To date, there are no biomarkers in blood and serum or cerebrospinal fluid (CSF) for detection, follow-up, or prognostication of gliomas.

Recent studies indicated that various microRNAs (miRNAs) play important roles in human cancer. MiRNAs are evolutionarily conserved. On average, they are only 22 nucleotides long noncoding RNA molecules capable of silencing gene expression at a post-transcriptional level by means of pleiotropic suppression of sequence-complementary mRNA targets.² Dependent

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on the target gene, miRNAs are able to function both as tumor suppressors and as oncogenes.²

In a previous study, we detected microRNAs in CSF and identified those molecules as markers for primary diffuse large B-cell lymphoma of the CNS (PCNSL).³ Because characteristic microRNA alterations (*miR-15a*, *miR-15b*, *miR-16*, *miR-19b*, *miR-21*, *miR-92*, *miR-106a*, *miR-155*, and *miR-204*) have also been identified in gliomas,^{4–7} we analyzed CSF samples from 10 patients with glioma. In this pilot study, we identified differentially expressed microRNAs in CSF samples from patients with glioma; these may serve as encouragingly sensitive and specific disease markers.

Material and Methods

Patient Characteristics and CSF Samples

From February 2009 through April 2011, consecutive CSF samples from patients with glioma ($n = 10$), control patients with various neurologic disorders ($n = 10$), patients with PCNSL ($n = 23$), and patients with brain metastases or leptomeningeal involvement of carcinoma ($n = 7$) that were sent to our laboratory for routine chemical and cellular diagnostics were analyzed. Two CSF samples from patients with glioma were collected intraoperatively after informed consent. The remaining CSF samples were collected before surgery, chemotherapy, and radiotherapy by diagnostic lumbar puncture after written informed consent and after exclusion of large intracranial mass lesions and/or increased intracranial pressure. The University of Bochum ethical committee approved the CSF sample collections. CSF samples were centrifuged ($500 \times g$, 10 min, room temperature) within 60 min after collection to remove cells and debris and were stored at -80°C until further processing. An exclusion criterion from further analyses was hemorrhagic CSF collection. Sample aliquots were used for routine CSF diagnostics, including quantification of biochemical markers, cytopathology, and immunophenotyping by flow cytometry, as recently reported.^{8,9}

RNA Extraction, Reverse-Transcription, and Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using a *mirVana* RNA isolation kit (Ambion) according to the manufacturer's instructions. In brief, CSF samples were thawed on ice, and 0.4 mL of CSF was diluted with an equal volume of *mirVana* PARIS 2 \times denaturing solution and subsequently incubated for 5 min on ice. Equal volumes of acid, phenol, and chloroform (Ambion) were added to each aliquot, and samples were subsequently centrifuged for 5 min at $10,000 \times g$. Next, glycogen was added to aqueous phases, which were subsequently mixed with 1.25 volumes of 100% ethanol. After passage through a *mirVana* PARIS column, several washing steps were performed according to the manufacturer's protocol. Finally, RNA was

recovered in 100 μL of elution buffer. The RNA concentration was determined by measuring a 2 μL aliquot on a NanoDrop ND-3300 Fluorospectrometer. Sequences of all miRNAs and primers used for specific amplification are listed at <http://www.mirbase.org> and <http://www.appliedbiosystems.com/>. TaqMan miRNA assays (Applied Biosystems) to quantify miRNA levels were applied as has previously been published.³ In brief, 10 μL of total RNA solution was used in reverse-transcription reactions (16°C for 30 min, 42°C for 30 min, and 85°C for 5 min, followed by 4°C). Real-time PCR was performed using a 7500 Real-Time PCR System according to the manufacturer's protocol (Applied Biosystems). Cycling conditions were as follows: 95°C for 10 min and 40 cycles of 15 s at 95°C and 60 s at 60°C . Fluorescent data were converted into cycle threshold (Ct) measurements using the 7500 SDS system software (version 1.2.3; Applied Biosystems). Each sample was run in duplicates. Mean Ct values and standard deviations were calculated for all miRNAs. *MiR-24* was selected from several control miRNAs. The amount of target miRNA was normalized relative to the amount of *miR-24* ($\Delta\text{Ct} = \Delta\text{Ct}_{miR} - \Delta\text{Ct}_{miR-24}$). Relative expression levels (REs) were reported as $2^{-\Delta\text{Ct}}$.

Statistics

All statistical analyses were performed using SPSS (version 19; SPSS) and GraphPad Prism (version 5.0, GraphPad Software). Groupwise comparisons of distributions of clinical and biological data were performed by applying 2-tailed Mann-Whitney *U* tests and Kruskal-Wallis tests with Dunn's multiple comparison. Results were considered to be statistically significant at $P < .05$.

Results

Patient Characteristics

In this study, CSF samples from 10 patients with glioma, 10 control patients with various neurologic disorders, 23 patients with PCNSL, and 7 patients with brain metastases or leptomeningeal involvement of carcinomas (3 breast cancers, 2 small cell lung cancers, 1 gastric cancer, and 1 adenocarcinoma of unknown primary) were analyzed. In 9 patients with glioma, a histopathologic diagnosis of glioma was established by brain biopsy. The biopsy for 1 patient could not be performed because of unfavorable tumor location. The diagnosis for this patient was based on unambiguous MRT findings. There were 3 patients with low-grade astrocytomas (World Health Organization [WHO] grade II), 2 patients with anaplastic astrocytoma (WHO grade III), and 5 patients with glioblastoma (WHO grade IV). At the time of CSF sample collection, the disease was newly diagnosed in the majority of patients with glioma ($n = 8$), and in 2 of 10 patients, progressive disease (progressive astrocytoma WHO grade II, 14 years after the initial diagnosis and a case of secondary

Table 1. miRNA expression in CSF 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-15a	34.81	0.50	36.71	0.77	.07
miR-15b	28.05	0.43	34.80	1.46	<.001
miR-16	26.28	0.82	28.69	1.26	.12
miR-19b	30.12	0.80	33.01	2.84	.08
miR-21	26.95	0.90	33.25	1.43	<.001
miR-24	28.90	0.56	31.18	1.00	.08
miR-92	28.30	0.66	29.68	0.25	.08
miR-106b	31.33	0.91	31.31	5.71	.62
miR-155	33.74	0.78	34.50	0.76	.81
miR-204	28.24	0.51	29.08	2.26	.26

^an = 10.^bPatients with miscellaneous neurologic disorders (n = 10)^cData are means of Ct values (groupwise).^dStandard deviation.^eThe P value is for comparison of miRNA expression among patients with glioma and control patients and was calculated using the Mann-Whitney U test.

glioblastoma after the first diagnosis of low-grade astrocytoma, 1 year previously) had been diagnosed. Leptomeningeal dissemination was detected in none of the patients with glioma. The glioma study population consisted of 5 male and 5 female patients; the age was 18–75 years (mean age, 51 years). The mean age of 3 patients with low-grade astrocytomas (WHO grade II) was 39 years. The clinical characteristics of control subjects and patients with PCNSL are reported elsewhere.³

Detection of miRNAs in CSF Samples from Patients with Glioma by qRT-PCR

First, we screened CSF samples collected from all patients with glioma (n = 10) and from control patients with

miscellaneous neurologic disorders (n = 10) (Table 1). A selection of potential CSF-based miRNA marker candidates for glioma was predicted on the basis of published miRNA expression data in glioma tissues^{4–7} and by our recent study on differentially expressed miRNAs in CSF samples from patients with PCNSL.³ This process resulted in 9 candidate miRNAs (*miR-15a*, *miR-15b*, *miR-16*, *miR-19b*, *miR-21*, *miR-92*, *miR-106a*, *miR-155*, and *miR-204*) for further investigation. On the basis of data from our former investigation,³ *miR-24* was selected as control with uniform expression levels and a sufficient abundance in CSF for normalization purposes.

We used TaqMan qRT-PCR assays for detection of miRNAs and, primarily, screened CSF samples collected from glioma patients (n = 10) and from control patients with miscellaneous neurologic disorders (n = 10) (Table 1). Results of this initial screening indicated that 2 of 9 candidate miRNAs (*miR-15b* and *miR-21*) showed significantly increased levels (decreased Ct values) in CSF samples from patients with glioma, compared to samples from control patients'. *MiR-15a*, *miR-16*, *miR-19b*, *miR-92*, *miR-106a*, *miR-155*, and *miR-204* were detected by qRT-PCR; however, expression levels were comparable in patients with glioma and control patients. Low-abundant expression of *miR-24* could be measured in all samples (Table 1).

Diagnosis of Glioma on the Basis of miR-15b and miR-21 Levels in CSF

After differential expression of *miR-15b* and *miR-21* in CSF samples from patients with glioma and control patients with miscellaneous neurologic disorders symptoms was found, we next compared expression of *miR-15b* and *miR-21* in CSF samples from patients with glioma with that in patients with PCNSL and with brain metastasis of carcinoma, which represent important differential diagnoses of glioma (Fig. 1). MiRNA expression data were normalized using

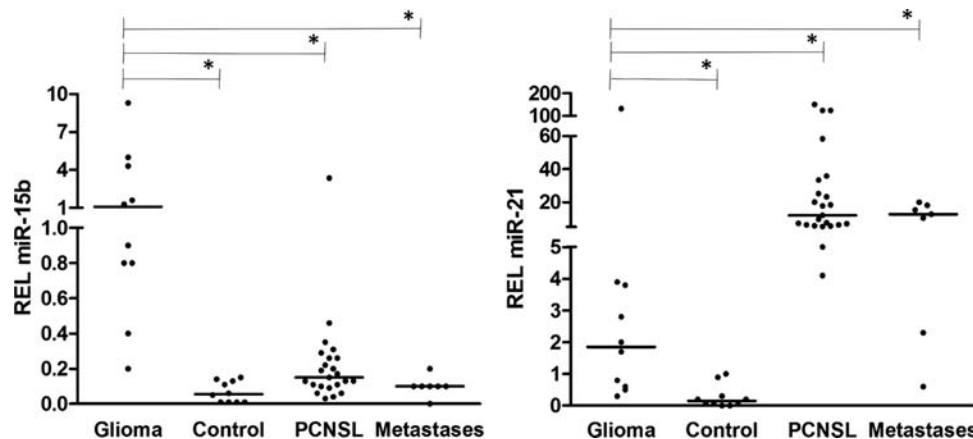


Fig. 1. Relative expression levels of *miR-15b* and *miR-21* in CSF samples from patients with glioma, control patients, and patients with PCNSL. Scatter plots of expression levels of *miR-15b* and *miR-21* in CSF samples from patients with glioma (n = 10), compared with subgroups of patients with miscellaneous (n = 10) CNS disorders with PCNSL (n = 23) and with brain or leptomeningeal metastases (n = 7). Relative expression levels (REL) of the miRNAs (y-axis) are normalized to *miR-24*. The black horizontal lines represent median REL values. Group-wise P values are indicated as determined in Kruskal-Wallis tests with Dunn's multiple comparisons (*P < .05).

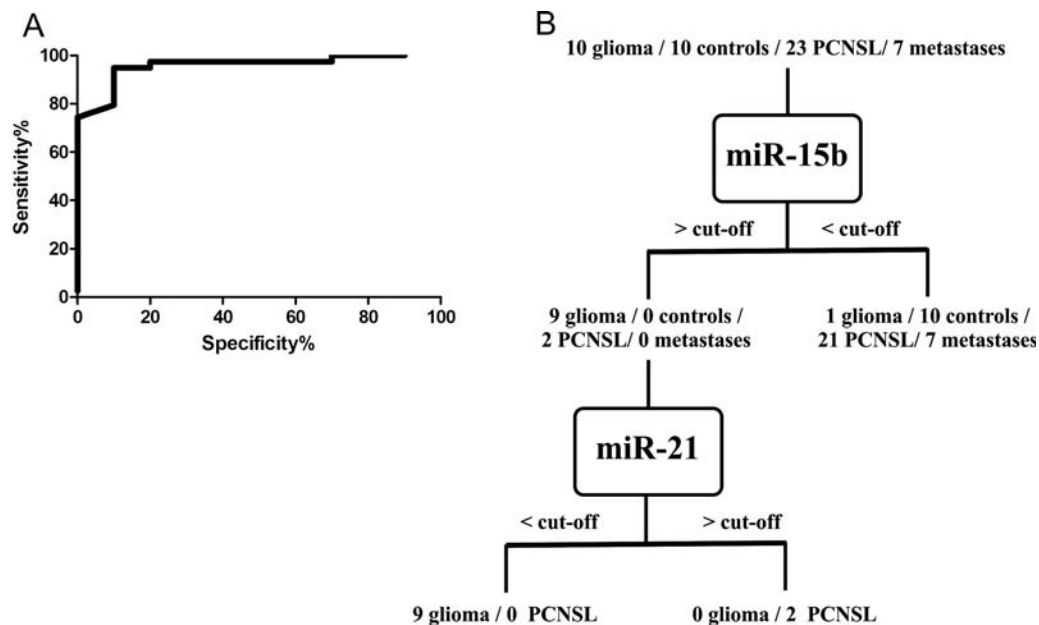


Fig. 2. Receiver-operating characteristic (ROC) curve analyses using relative expression level of *miR-15b* in CSF and classification tree based on expression of *miR-15b* and *miR-21* in CSF. (A) CSF relative expression of *miR-15b* as a single biomarker yielded an area under the curve (AUC) of 0.96 (95% confidence interval, 0.925–1.021). (B) CSF miRNA expression classification tree of correct diagnosis in 90% of patients with glioma and 100% of patients without glioma. Relative expression cutoff levels of ≥ 0.4 REL for *miR-15b* and ≤ 8.0 REL for *miR-21*, respectively, were applied for diagnostic placements as depicted.

miR-24 levels in individual CSF specimens and reported as RELs, as recently detailed.³

Comparison of CSF expression of 2 candidate miRNAs in patients with glioma and control patients exhibited significant differences for *miR-21* and *miR-15b*. Increased mean REL of *miR-21* and *miR-15b* were demonstrated in CSF samples from patients with glioma (Fig. 1). On the other hand, comparison of CSF collected from patients with glioma and PCNSL revealed significantly higher expression of *miR-15b* and, of note, decreased expression of *miR-21* in CSF samples from patients with glioma in 2-tailed Mann-Whitney *U* tests (Fig. 1).

Next, receiver-operating characteristic (ROC) curves of *miR-15b* were plotted. We observed clear separations between the groups of patients with glioma and those without glioma, including control subjects and patients with PCNSL, with an area under the curve (AUC) of 0.96. Corresponding to this analysis, cutoff CSF REL with the highest accuracy for *miR-15b* was determined as follows: REL of 0.4 with 90% sensitivity and 94.9% specificity (Fig. 2A). The only false-negative result was observed in the patient who received a diagnosis of anaplastic astrocytoma (WHO grade III). To increase the specificity of discrimination of glioma from other diseases, we combined REL of *miR-15b* with *miR-21* and developed a diagnostic tree (Fig. 2B). For *miR-21*, an REL of 8.0 was used on the basis of our former study with CSF samples from the same control subjects and patients with PCNSL.³ In summary, all but one patient with glioma could be precisely identified, considering REL above the cutoff for *miR-15b* and under the cutoff for *miR-21*. Furthermore, all patients without glioma were identified correctly with REL below the cutoff

for *miR-15b* and above the cutoff for *miR-21*. In summary, the miRNA diagnostic tree exhibited excellent sensitivity and specificity in our cohort of 10 patients with glioma, 10 control subjects, 23 patients with PCNSL, and 7 patients with brain metastases (Fig. 2A).

Discussion

To our knowledge, this is the first observation of deregulated microRNA expression in CSF samples from patients with glioma. Our results demonstrate that miRNAs circulating in CSF may serve as novel biomarkers for the detection of glioma. We found that the levels of *miR-15b*, as measured in qRT-PCR assays, were significantly increased in CSF samples from patients with glioma, compared with control subjects with miscellaneous neurologic disorders or patients with PCNSL or brain metastases.

MiR-15b had a significant diagnostic value for glioma and yielded an AUC of 0.96 in ROC analyses. Inclusion of *miR-15b* and *miR-21* in combined expression analyses further increased the discriminatory diagnostic value. In summary, our study of combined *miR-15b* and *miR-21* analyses demonstrated that CSF levels of miRNA could accurately distinguish patients with glioma from control subjects, including patients with PCNSL and brain metastases or leptomeningeal carcinomas.

In our study, *miR-15b* was the most abundant miRNA in CSF samples from patients with glioma. *MiR-15b* showed 25-fold higher mean expression levels in CSF samples from patients with glioma than in control subjects, 5-fold higher mean expression levels than in patients with PCNSL, and 17-fold higher mean expression than in

patients with brain metastases or leptomeningeal carcinoma. These data are consistent with those from Xia et al. and Guan et al., who demonstrated high *miR-15b* expression levels in glioma tissue.^{5,6} *MiR-15b*, a member of *miR-15/16* family, has been reported to play an important role in glioma carcinogenesis by regulating cell cycle progression.⁶ Of interest, Xia et al. suggested that *miR-15b* may function as a tumor suppressor, inducing cell cycle arrest in G1 phase by targeting Cyclin E1 (CCNE1).⁶ CCNE1 is a positive regulator of the cell cycle by controlling the transition of cells from G1 to S phase. In contrast to glioma, *miR-15b* has been reported to be down-regulated in CLL and in gastric cancer.^{10,11}

MiR-21, which also has been found to be overexpressed in CSF samples from patients with glioma, compared with control subjects, is one of the most consistently expressed microRNAs in cancer arising from other tissues. *MiR-21* functions as an oncogene in glioma, preventing apoptosis.⁵ It targets tumor suppressor genes, such as PTEN or *PDCD4*, and modulates the networks of p53 and transforming growth factor β .¹² *MiR-21* has also been described to promote tumor invasion by directly targeting and downregulating anti-invasive proteins, such as TIMP3.¹³ Of note, *miR-15b* and *miR-21* were found to be expressed at significantly higher levels in glioblastomas than in anaplastic astrocytomas.⁶ On the other hand, there was no significant association of survival among patients with glioma and expression levels of *miR-21* or *miR-15b*.⁶ In our study, we found no association of expression levels of *miR-15b* and *miR-21* with the glioma grading or with the survival among patients with glioma. However, this negative finding has to be interpreted in light of the small sample size of our pilot study and has to be reevaluated in a larger patient cohort.

In summary, this study provides the first evidence of significant miRNA levels in CSF samples from patients with glioma. Considering the high diagnostic value of combined *miR-15b* and *miR-21* analyses in this pilot

study, we anticipate miRNA analyses to have a potential to characterize CSF markers for glioma. With regard to a lack of biomarkers in gliomas thus far, the availability of miRNA, such as *miR-15b* and *miR-21*, might facilitate the diagnosis and clinical management of this cancer type. Moreover, there is a possibility that microRNA in CSF may be used as indicators of treatment response and markers for disease prognosis. A relevant limitation of our pilot study is the small sample size of patients with glioma. This is reflecting, for the most part, the fact that lumbar punctures and CSF analyses are, at present, not a standard diagnostic procedure for suspected glioma. Undoubtedly, our finding of *miR-15b* and *miR-21* as valuable CSF biomarkers for differentiation of glioma and PCNSL has to be validated in a larger patient study. However, our results provide the clear rationale for future investigations, preferably in prospective trials, of miRNAs in the CSF for diagnostic and prognostic purposes in brain tumors, such as glioma. It is likely that, by both qRT-PCR assays and miRNA array technologies, CSF-based miRNA markers that are specific for different brain tumors (eg, glioma subtypes) will be discovered in the future.

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Conflict of interest statement. None declared.

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