Identification of microRNAs in the cerebrospinal fluid as marker for primary diffuse large B-cell lymphoma of the central nervous system

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ABSTRACT

The diagnosis of primary central nervous system lymphoma (PCNSL) depends on histopathology of brain biopsies, since disease markers in the cerebrospinal fluid (CSF) with sufficient diagnostic accuracy are not available yet. MicroRNAs (miRNAs) are regulatory RNA molecules that are deregulated in many disease types including cancer. Recently, miRNAs have shown promise as markers for cancer diagnosis. In the current study, we demonstrate that miRNAs are present in the CSF of patients suffering from PCNSL. Making use of a candidate approach and of miRNA quantification by RT-PCR, miRNAs with significant levels in the CSF of PCNSL patients were identified. *MiR-21*, *miR-19*, and *miR-92a* levels in CSF collected from PCNSL patients and controls with inflammatory CNS disorders and other neurologic disorders indicated a significant diagnostic value of this method. Receiver-operating characteristic analyses revealed area under the curves of 0.94, 0.98, and 0.97 for *miR-21*, *miR-19*, and *miR-92a* CSF levels in discriminating PCNSL from controls. More importantly, combined miRNA analyses resulted in an increased diagnostic accuracy with 95.7% sensitivity and 96.7% specificity. We also demonstrated a remarkable stability of miRNAs in the CSF. In conclusion, CSF miRNAs are potentially useful tools as novel non-invasive biomarker for the diagnosis of PCNSL.
INTRODUCTION

Unraveling the etiology of focal brain lesions in patients with unexplained neurologic symptoms remains a clinical challenge. Especially in patients with primary central nervous system lymphoma (PCNSL), definitive diagnosis often is not possible based on radiographic features and responsiveness to corticosteroids, which both do not specifically distinguish between lymphoma and inflammatory central nervous system (CNS) disease. In most patients with suspected CNS lymphoma who present with rapidly deteriorating neurologic symptoms, stereotactic brain biopsy remains the diagnostic procedure of choice. However, CNS biopsies are associated with the risk of hemorrhage and neurologic damage, and a definitive histopathologic diagnosis can not always be achieved.

Since PCNSL represent highly aggressive tumors, early diagnosis is essential for successful treatment and improvement of disease prognosis. Although evaluation of the cerebrospinal fluid (CSF) is less invasive than brain biopsy, cytopathologic, immunophenotypic, and genetic analyses of CSF cells are much less sensitive. Protein markers within the CSF include antithrombin, soluble CD27, and free immunoglobulin light chains. They have been demonstrated helpful with improved diagnostic sensitivities, however, their utility in accurate diagnosis of PCNSL from the CSF has not finally been established.

MicroRNAs (miRNAs) are small regulatory RNA molecules that bind the 3’-untranslated regions of mRNA transcripts and inhibit gene expression at a post-transcriptional level via interference with translational initiation or degradation of mRNA. In several studies, miRNAs have been shown to play key regulatory roles in a wide range of genetic pathways.
controlling cellular differentiation, proliferation, and apoptosis in physiologic conditions as well as different human diseases. There is increasing evidence that dysfunctional expression of miRNAs is a common feature of many types of cancer, and, miRNAs play a direct role in cancer as they can function as oncogenes and tumor suppressors. Deregulated miRNA expression is found in various malignancies including leukemia and lymphoma.

MiRNAs are increasingly utilized as markers for diagnostic and prognostic purposes. Expression analyses of miRNAs can be accomplished directly from tumor samples. Furthermore, circulating miRNAs stably packaged in microvesicles can be detected in human serum and plasma. Previously, it has been demonstrated that high expression levels of distinctive miRNAs are detectable in sera from patients with different types of cancers and are related to disease prognosis, e.g. B-cell lymphoma, prostate cancer, and non-small cell lung cancer. Until today, there is no report of miRNAs identified in the CSF of patients with primary diffuse large B-cell lymphomas of the CNS.

In this study, we hypothesized that miRNAs could be useful CSF-based markers for detection of PCNSL. A candidate miRNA approach assessing miRNA expression by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) in the CSF of PCNSL patients and control subjects with different neurologic disorders was chosen to evaluate the potential of miRNAs as a novel class of genetic CSF marker for PCNSL.
MATERIAL AND METHODS

Patient characteristics and CSF samples

Between February 2009 and August 2010, consecutive CSF samples from patients with primary CNS lymphoma (n=23) and control patients with various neurologic disorders (n=30; patient details listed in supplementary Tab. S1, S2A, S2B) sent to our laboratory for routine chemical and cellular diagnostics were analyzed. CSF samples were collected prior to chemotherapy by diagnostic lumbar puncture after written informed consent and after exclusion of large intracranial mass lesions and/or increased intracranial pressure. All patients were seronegative for HIV. The University of Bochum ethical committee had approved CSF sample collections.

CSF samples were centrifuged (500 x g, 10 min, room temperature) within 60 minutes of 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.

RNA extraction, reverse-transcription and quantification by real-time polymerase reaction

Total RNA was extracted using a mirVana RNA isolation kit (Ambion, Austin, USA) according to the manufacturer’s instructions. Briefly, CSF samples were thawed on ice and 0.4 ml
of CSF was diluted with an equal volume of mirVana PARIS 2X denaturing solution, and subsequently incubated for 5 min on ice. Equal volumes of acid/phenol/chloroform (Ambion) were added to each aliquot, samples were subsequently centrifuged for 5 min at 10,000 x 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 carried out following 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, Foster City, USA) to quantify miRNA levels were applied as has previously been published 18. In brief, 10 µl of total RNA solution 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 on a 7500 Real-Time PCR System according to the manufacturer’s protocol (Applied Biosystems). Cycling conditions were as follows: 95°C for 10 min, 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Fluorescent data were converted into cycle threshold (Ct) measurements by the 7500 SDS system software (version 1.2.3; Applied Biosystems). Each sample was run in duplicates. Mean cycle threshold (Ct) values and standard deviations were calculated for all miRNAs. MiR24 was selected from several control miRNAs, as it showed uniform expression levels. The amount of target miRNA was normalized relative to the amount of miR-24 ($\Delta$Ct = $\Delta$Ct$_{miR}$ - $\Delta$Ct$_{miR-24}$). Relative expression levels (REL) were reported as $2^{-\Delta\text{Ct}}$ (Tab. S1, S2A, S2B).
To determine stability of miRNAs, miR-21 was quantified in CSF samples stored at room temperature (22°C) for a maximum of 4 days. CSF samples from five different PCNSL patients were thawed and aliquoted into separate tubes. RNA was isolated immediately after thawing or following 24 h, 48 h, and 96 h of storage, respectively. To evaluate the resistance of CSF miRNA to cleaving enzymes, an RNase mixture (Ambion, RNase Cocktail™) was added to 500 µl of CSF at a final concentration of 5 units/ml RNase A and 200 units/ml RNase T1 and then incubated at 37 °C for 30 min. MiR-30a-5p and miR-24 in a mixture of isolated miRNA were used as positive controls. MiRNAs were purified using the mirVana miRNA isolation kit (Ambion) and measured in qRT-PCR as detailed above.

**Statistics**

All statistical analyses were performed using SPSS (version 18) and GraphPad Prism (version 5.0). Groupwise comparisons of distributions of clinical and biological data were performed applying two-tailed Mann-Whitney U-tests and Kruskal-Wallis tests with Dunn’s multiple comparison. Results were considered statistically significant at p-values < 0.05. Retest reliabilities were assessed using the intraclass-correlation coefficient (ICC). A two way mixed type consistency ICC was chosen. ICC interpretation was similar to Cohen's Kappa for inter-rater reliability (ICC <0.20: poor, 0.20-0.39: fair, 0.40-0.59: moderate, 0.60-0.79: substantial agreement, >0.80: outstanding inter-rater reliability).
RESULTS

Patient characteristics

In this study, CSF samples from 23 patients with primary CNS lymphomas (Tab. S1) and 30 control patients with various neurologic disorders including CNS inflammation (n=20) were analyzed (Tab. S2A, S2B). The PCNSL study population consisted of fourteen male and nine female patients; age was between 42 and 77 years (mean age 64 years). In all 23 PCNSL patients, a histopathologic diagnosis of diffuse large B-cell type lymphoma was established by brain biopsies. At the time of CSF collection, the disease was newly diagnosed in the majority of PCNSL patients (n=19; 82.6%), in four (17.4%) of 23 patients a relapse of CNS lymphoma had been diagnosed. Leptomeningeal lymphoma dissemination was detected by concordant findings of cytopathology and flow-cytometry, as recently reported 6,11, in four (17.4%) of all 23 PCNSL patients (Tab. S1). Twelve (52.2%) of 23 PCNSL patients were treated with corticosteroids at the time of CSF sample collection.

Detection of miRNAs in the CSF of PCNSL patients by qRT-PCR

First, we asked whether miRNAs were detectable in frozen CSF samples collected from PCNSL patients (n=23; Tab. S1). In direct measurements of total RNA utilizing a fluorospectrometer, low RNA concentrations were detected in the CSF specimens (mean 1.8 ng/ml; SD 0.8); no obvious differences of total RNA concentrations among several CSF specimens from individuals with and without neurological disorders were found (data not shown). Consider-
ing these results, we chose a candidate approach measuring miRNAs in the CSF exploiting specific miRNA amplification by means of qRT-PCR reactions.

We reasoned that a suitable disease indicator for PCNSL would be (i) expressed by lymphoma cells at moderate or high levels, and (ii) would be present at low or undetectable concentrations in CSF derived from control patients. A selection of potential CSF-based miRNA marker candidates for PCNSL was obtained by compiling miRNAs expressed in diffuse large B-cell lymphomas and primary CNS lymphomas, based on published miRNA expression data in lymphoma tissues. This process resulted in six candidate miRNAs (miR-15b, miR-19b, miR-21, miR-92a, miR-106b, miR-204) for further investigation. Four miRNAs (miR-24, RNU48, RNU6b, RNU44) were selected as controls with likely uniform expression levels and a sufficient abundance in CSF for potential normalization purposes.

We employed TaqMan qRT-PCR assays for detection of miRNAs and, primarily, screened CSF samples collected from all PCNSL patients (n=23; Tab. S1) and a subset of control patients with miscellaneous neurologic disorders (n=10; Tab. S2A). Results of this initial screen indicated that three of six candidate miRNAs (miR-21, miR-19b, miR-92a) showed significantly increased levels (decreased Ct-values) in the CSF of PCNSL patients compared to control patients’ CSF, respectively (Tab. 1). MiR-15b, miR-106b, and miR-204 were detected by qRT-PCR; however, expression levels were comparable among PCNSL and control patients (Tab. 1). Control microRNAs RNU48, RNU6b, RNU44 were not detectable in the CSF by qRT-PCR (mean Ct-values > 40.0, data not shown), while low-abundant expression of miR-24 could be measured in all samples (Tab. 1). Since miR-24 was not expressed in different levels in PCNSL and control patients, it was chosen for normalization of miRNA expression levels in subsequent analyses of CSF miRNA expression in a larger cohort of control patients.
Diagnosis of PCNSL based on miR-19b, miR-21, and miR-92a levels in the CSF

Important differential diagnoses of PCNSL include demyelinating and inflammatory CNS diseases, such as multiple sclerosis. Having demonstrated differential expression of miRNAs in the CSF of PCNSL patients and control patients with miscellaneous neurologic disorders/symptoms, we next analyzed expression of miR-15b, miR-19b, miR-21, miR-92a, miR-106b, miR-204 in the CSF of patients with different inflammatory CNS diseases (Tab. S2A, S2B). MiRNA expression data were normalized utilizing mi-R24 levels in individual CSF specimens and reported as relative expression levels (REL). Comparison of CSF expression of all six candidate miRNAs among PCNSL patients (n=23) and all controls (n=30) revealed significant differences for miR-21, miR-19b, miR-92a, and miR-15b in two-tailed Mann-Whitney tests, respectively (Fig. 1A). Increased mean and median REL of miR-21, miR-19b, and miR-92a were demonstrated in PCNSL patients’ CSF. For miR-15b, the mean expression level was significantly reduced in CSF of patients with PCNSL (mean REL 0.60) as compared to control patients (mean REL 0.91). However, the median miR-15b REL was increased in PCNSL patients. This result indicated that the mean value for miR-15b was considerably influenced by outlying values in the control group. Accordingly, the expression profile of miR-15b needs to be further addressed in a larger cohort to clarify the meaning of outlying miR-15b expression in a subset of patients with inflammatory CNS disease. Considering only those miRNAs (miR-21, miR-19b, miR-92a) showing increased mean and median REL in PCNSL patients, significant differences in REL were also found among subgroups (PCNSL vs. inflammation, PCNSL vs. miscellaneous) as determined by Kruskal-Wallis tests including Dunn’s multiple comparison analyses (Fig. 1B).
Next, relative expression levels (REL) of all six candidate marker miRNAs were analyzed groupwise and receiver-operating characteristics (ROC) curves were plotted. As depicted in Fig. 2, excellent separations between the groups of PCNSL and control patients were observed for miR-21, miR-19b, and miR-92a, with areas under the curve (AUC) of 0.94, 0.98, and 0.97, respectively. On the contrary, AUC for miR-15b, miR-106b, and miR-204 were calculated 0.85, 0.60, and 0.65, respectively. Based on these analyses, cut-off CSF REL with the highest accuracy were determined for miR-21, miR-19b, and miR-92a as follows: miR-21 8.0 REL (95.7% sensitivity; 83.3% specificity), miR-19b 1.4 REL (95.7% sensitivity; 83.7% specificity), miR-92a 2.5 REL (95.7% sensitivity; 80.0% specificity).

In order to distinguish PCNSL from other diseases based on CSF miRNA expression more specifically, we combined miR-21, miR-19b, and miR-92a REL and developed a diagnostic tree (Fig. 3, Tab. 2). In summary, 22 (95.7%) of 23 PCNSL patients could be correctly identified considering REL above the cut-offs for miR-21 and for one of each miR-19b or miR-92a, respectively. On the contrary, only one control patient suffering from multiple sclerosis (patient 15, Tab. S2B) was falsely allocated to the PCNSL group with REL above the cut-offs for miR-21 and miR-92a. Overall, the specificity of the miRNA diagnostic tree was 96.7% in our cohort of 23 PCNSL and 30 control patients (Fig. 3).

Reproducibility of miRNA quantification and miRNA stability in CSF

Considering the potential of miRNAs as novel marker in the CSF, we next analyzed the reproducibility of measurements in TaqMan qRT-PCR assays. Levels of miR-21, miR-19b, and miR-92a were measured twice in the same CSF samples collected from each of six PCNSL patients. For miR-21, the Ct-values ranged between 21.4 and 27.6, for miR-19b Ct-values...
between 26.9 and 30.7 were measured, and for miR-92a Ct-values in the range of 24.6 and 29.8 were determined by TaqMan qRT-PCR. In a second set of measurements, Ct-values for each miRNA were re-analyzed in each patient applying the same experimental conditions. Statistical analyses among independent measurements verified outstanding retest reliabilities as indicated by the following intra-class correlation coefficients (ICC): miR-21 ICC 0.98 (95% confidence interval (CI) 0.89-0.99, p<0.001), miR-19b ICC 0.98 (95% CI 0.88-0.99, p<0.001), miR-92a ICC 0.97 (95% CI 0.78-0.99, p=0.001).

An important requirement for the utility of miRNAs as markers is their stability in CSF samples ex vivo. First, we investigated the stability of miR-21, miR-19b, and miR-92a in CSF by subjecting specimens to up to ten cycles of freeze-thawing. This procedure had minimal effect on miRNA levels as determined by qRT-PCR (data not shown). Prior reports have suggested that miRNAs in plasma exist in a form that is resistant to intrinsic RNase activity 16. In the CSF, low RNase activities are present 22. To address the question whether miRNAs in CSF exist in a form that is resistant to ribonucleases, we measured levels of miR-21 in CSF specimens following incubation with RNase. While levels of extrinsic miRNAs which had been added to the CSF decreased significantly after RNase exposure, the concentrations of endogenous miRNAs (miR-21, miR-19b, and miR-92a) in the CSF were not altered by ribonucleases (data not shown). We concluded that endogenous miRNAs in the CSF exist in a form resistant to RNase cleavage, a finding which was in line with those for serum and plasma miRNAs 16.

Next, we asked whether miRNAs are stable in stored CSF samples. Accordingly, CSF samples collected from five PCNSL patients were kept for up to 96 h at room temperature. Expression levels of miRNAs were measured immediately and after 24 h, 48 h, and 96 h of stora-
rage in TaqMan qRT-PCR assays. We found miR-21 levels in stored CSF specimens to be stable over time (data not shown). Here, Ct-values at 0 h ranged between 23.4 and 25.6 (mean Ct 24.9). At 24 h and 48 h, absolute decreases of mean CT-values of 0.06 (ΔCt% 0.23) and 0.38 (ΔCt% 1.52) were observed, respectively. At the maximum CSF sample storage time of 96 h at room temperature, the mean absolute CT-value decreased by 1.39 (ΔCt% 5.58). In summary, we concluded that miRNAs exist in a stable form in CSF.
DISCUSSION

Several reports have described that deregulation of miRNAs is tightly linked to cancer. In addition, some miRNAs are closely associated with the clinical course of malignant tumor disease. Hence, expression analysis of miRNAs is of increasing interest for diagnostic and prognostic purposes. Evaluation of miRNA expression in primary tumor cells and tissues has been the basis of most studies in the field. Recently, it has been discovered that tumor derived miRNAs are present in extracellular body fluids such as serum and plasma. Most of the miRNAs circulating in peripheral blood are included in lipid or lipoprotein complexes and, therefore are highly stable. Considering these findings, we raised the question whether (i) miRNAs are detectable in the extracellular liquid compartment of the CSF in PCNSL patients and, whether (ii) miRNA detection in CFS may facilitate the diagnosis of PCNSL. CSF-based miRNA studies are in their early infancy. To date, only one study has reported that miRNAs can be measured in the CSF collected from healthy individuals and Alzheimer patients.

In our study, a candidate approach was chosen and miR-21, miR-19, miR-92a, miR-15b, miR-106b, and miR-204 were selected as miRNAs putatively over-expressed in CSF of PCNSL patients. We found that the levels of miR-19b, miR-21, and miR-92a, as measured in RT-PCR assays, were significantly increased in CSF samples from PCNSL patients as compared to controls with inflammatory CNS disease or other neurologic disorders. MiR-21, miR-19, and miR-92a had a significant diagnostic value for PCNSL and yielded AUC of 0.94, 0.98, and 0.97 in ROC analyses. Inclusion of these three miRNAs in combined expression analyses further increased their discriminatory diagnostic value. In summary, our study of combined
miR-21, miR-19b, and miR-92a analyses in PCNSL patients demonstrated that CSF levels of miRNA could distinguish, with considerable specificity (96.7%) and sensitivity (95.7%), patients with PCNSL in contrast to other neurologic disorders, most importantly inflammatory CNS diseases.

In our study, miR-21 was the most abundant miRNA in CSF of PCNSL patients. Mean relative expression of miR-21 was 60.0 in CSF of PCNSL as compared to 3.8 in CSF of controls. These data are consistent with Lawrie et al. 21 and Robertus et al. 20 who showed high miR-21 expression levels in samples of diffuse large B-cell lymphoma including PCNSL. Interestingly, miR-21 has been shown to be expressed in a variety of tumors and to be associated with the downregulation of bcl-2 and PTEN. Both, miR-19b and miR-92a are members of the poly-cistronic microRNA-17~92 cluster located on human chromosome 13 13. In the present study, miR-19b showed 14-fold higher mean expression levels and, miR-92a was 10-fold higher expressed in CSF of PCNSL patients in comparison to control patients. Previously, evidence has been provided that the microRNA-17~92 cluster is frequently over-expressed in B-cell lymphoma cell lines, the majority of diffuse large B-cell lymphomas 12, and also in PCNSL 20. Thus, the role of microRNA-17~92 in lymphoma is further highlighted by our findings of differential expression of miR-19b and miR-92a in the CSF of PCNSL patients.

Normalization is an important step for accurate quantification of RNA levels with qRT-PCR. For miRNA, circulating in extracellular body fluids including those detected in the CSF, no consensus internal controls have been established yet. In our study, the yields of total RNA prepared from small volume CSF were below the limit of accurate quantification by fluorospectrometry. These results are confirmed in a study by Cogswell et al. 23, and are similar to findings reported for plasma and serum 16. Accordingly, absolute RNA concentrations could
not be utilized for normalization purposes in our measurements. We evaluated four miRNAs (RNU48, RNU6b, RNU44, miR-24) and identified miR-24 to exhibit consistent expression across patients and controls. Prior to our study, miR-24 has been reported as appropriate miRNA control for normalization purposes. Accordingly, miR-24 was chosen as normalizing control miRNA in our current study.

Extracellular miRNAs circulating in the peripheral blood are obviously included in cell membrane derived particles, such as apoptotic bodies, microvesicles, and exosomes. There is evidence that packaging of miRNA into these particles results in protection from endogenous ribonucleases in plasma/serum. In agreement with these findings, miRNAs in the CSF exhibited a remarkable stability as demonstrated here in exposure to exogenous RNase, repeated freeze-thaw cycles, and long-term storage of CSF specimens in our experiments. Possible explanations for this phenomenon include miRNA protection in exosomes or association of miRNA with other molecules, e.g. CSF proteins. Because the secretory mechanisms as well as the meaning of the existence of extracellular miRNAs remain largely unknown, additional studies exploring a potential biological function of miRNAs circulating in body fluids such as peripheral blood and CSF are required. Notably, the striking stability of CSF miRNA as compared to the fragility of cells within the CSF represents an important diagnostic advantage.

During the study period, 26 patients were referred to our institution for diagnosis and treatment of PCNSL. The majority of our patients (n=24; 92.3%) had CNS lesions not accompanied by major alterations in CSF kinetics nor a hydrocephalus preventing us from lumbar puncture. However, in two patients (7.7%) lumbar puncture was omitted given that space occupying cerebellar lesions were demonstrated in the posterior fossa. One patient had al-
ready undergone a lumbar puncture without complication in the referring hospital. In summary, in the greater group of our patients lumbar puncture was possible without a risk due to disturbed CSF kinetics or intracranial pressure. This finding was in line with those reported in the literature 24 and supports the potential diagnostic value of CSF analyses such as miRNA detection in PCNSL patients.

In conclusion, our results suggest that miRNA detected in the CSF can serve as marker for PCNSL as a model CNS disease. We anticipate that the results of our study are expanded by detection of other miRNAs in the CSF related to other neoplastic and inflammatory CNS disease. By means of both, qRT-PCR assays and miRNA array technologies, CSF-based miRNA markers that are specific for particular CNS disorders may be discovered in the future. Although the number of patients and controls studied here is still small, our study provides the rationale for future investigations of miRNAs in the CSF for diagnostic and prognostic purposes in a variety of CNS disease including PCNSL.
ACKNOWLEDGEMENT

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AUTHOR CONTRIBUTION


CONFLICT OF INTEREST / DISCLOSURES

The authors declare no conflict of interest and no competing financial interests.
REFERENCES


Table 1  miRNA expression in CSF of PCNSL versus control patients

<table>
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<th>miRNA</th>
<th>PCNSL patients(^1)</th>
<th>Control patients(^2)</th>
<th>p-value</th>
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<td></td>
<td>Ct  (^3)</td>
<td>SD (^4)</td>
<td>Ct</td>
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<td>miR - 21</td>
<td>26.29</td>
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<td>33.25</td>
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<td>miR - 19b</td>
<td>28.76</td>
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<td>miR - 92a</td>
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<td>1.17</td>
<td>30.24</td>
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<td>miR - 106b</td>
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<td>miR - 204</td>
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<tr>
<td>miR - 24</td>
<td>31.21</td>
<td>1.09</td>
<td>31.18</td>
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\(^1\) PCNSL patients (n=23)  
\(^2\) patients with miscellaneous neurologic disorders (n=10)  
\(^3\) Data are means of CT values (groupwise)  
\(^4\) standard deviation  
\(^5\) The p-value is for comparison of miRNA expression among PCNSL patients and control patients and was calculated using the Mann-Whitney U-Test.
### Table 2

<table>
<thead>
<tr>
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<th>miRNA – 21</th>
<th>miRNA – 19</th>
<th>miRNA - 92a</th>
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<td>≥ 8.0 REL*</td>
<td>≥ 1.4 REL</td>
<td>≥ 2.5 REL</td>
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<td><strong>Inflammation</strong></td>
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<td>1 / 20 (5.0)</td>
<td>3 / 20 (15.0)</td>
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<tr>
<td><strong>Miscellaneous</strong></td>
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</table>

* REL – relative expression level
**Figure Legends**

**Figure 1:** Relative expression levels of miRNAs in CSF of PCNSL and control patients

**Figure 1A:**

Scatter plots of expression levels of $\text{miR-21}$, $\text{miR-19b}$, $\text{miR-92a}$, $\text{miR-15b}$, $\text{miR-106b}$, and $\text{miR-204}$ in CSF samples from 23 PCNSL patients compared to 30 control patients with various neurologic disorders. Relative expression levels (REL) of miRNAs (y-axis) are normalized to $\text{miR-24}$. The black horizontal lines represent median REL values. P-values are indicated as determined in Mann-Whitney U statistical comparisons.

**Figure 1B:**

Scatter plots of expression levels of $\text{miR-21}$, $\text{miR-19b}$, and $\text{miR-92a}$ in CSF samples from PCNSL patients (n=23) compared to subgroups of patients with inflammatory (n=20) and miscellaneous (n=10) CNS disorders. Relative expression levels (REL) of the miRNAs (y-axis) are normalized to $\text{miR-24}$. 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 (* indicate p < 0.05).
Figure 2: Receiver-operating characteristics (ROC) curve analyses using relative expression levels of CSF miRNAs for discrimination of PCNSL and control patients

CSF relative expression of miR-21 yielded an AUC of 0.94 (95% CI 0.886-1.00), for miR-19b an AUC of 0.98 (95% CI 0.955-1.01), for miR-92a an AUC of 0.97 (95% CI 0.925-1.01), for miR-15b an AUC of 0.85 (95% CI 0.748-0.959), for miR-106b an AUC of 0.60 (95% CI 0.452-0.765), for miR-204 an AUC of 0.65 (95% CI 0.497-0.803), respectively.

Figure 3: CSF miRNA expression classification tree correctly diagnosing 95.7% of PCNSL and 96.7% of control patients

Relative expression cut-off levels of ≥ 8.0 REL for miR-21, ≥ 1.4 REL for miR-19b, and ≥ 2.5 REL for miR-92a, respectively, were applied for diagnostic placements as depicted.
Figure 1A
Figure 1B
Figure 2
Figure 3

23 PCNSL / 30 controls

\[ \text{miR-21} \]

\( \geq \text{cut-off} \) \hspace{1cm} < \text{cut-off} \\

22 PCNSL / 6 controls \hspace{1cm} 1 PCNSL / 24 controls

\[ \text{miR-19b/92a} \]

\( \geq \text{cut-off} \) \hspace{1cm} < \text{cut-off} \\

22 PCNSL / 1 control \hspace{1cm} 0 PCNSL / 5 controls

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Identification of microRNAs in the cerebrospinal fluid as marker for primary diffuse large B-cell lymphoma of the central nervous system

Alexander Baraniskin, Jan Kuhnenn, Uwe Schlegel, Andrew Chan, Martina Deckert, Ralf Gold, Abdelouahid Maghnouj, Hannah Zöllner, Anke Reinacher-Schick, Wolff Schmiegel, Stephan A. Hahn and Roland Schroers