Differential micro-RNA expression in primary CNS and nodal diffuse large B-cell lymphomas

Lars Fischer, Michael Hummel, Agnieszka Korfel, Dido Lenze, Korrina Joehrens, and Eckhard Thiel

Medical Clinic for Hematology and Oncology, Charité Universitätsmedizin, Berlin, Germany (L.F., A.K., E.T.) and Institute of Pathology, Charité Universitätsmedizin, Berlin, Germany (M.H., D.L., K.J.)

Most primary CNS lymphomas (PCNSL) are diffuse large B-cell lymphomas (DLBCL). However, clinical behavior and prognosis differ considerably from those for nodal DLBCL (nDLBCL), and their pathogenesis is still not fully understood. Micro-RNAs (miRNAs) have been associated with cancer development and progression. We investigated a large miRNA panel for differential expression in PCNSL and nDLBCL, to determine new mechanisms potentially involved in PCNSL pathogenesis. Using paraffin-embedded biopsy specimens from 21 HIV-negative patients with newly diagnosed PCNSL (n = 11) and nDLBCL (n = 10), we measured the expression of 365 miRNA species by quantitative real-time PCR using low-density PCR arrays. We found that 18 miRNAs were differentially expressed: median expression levels of 13 miRNAs were 2.1–13.1 times higher in PCNSL, and median expression levels of 5 miRNAs were 2.6–3.3 times higher in nDLBCL. MiRNAs upregulated in PCNSL were associated with the Myc pathway (miR-17-5p, miR-20a, miR-9), with blocking of terminal B-cell differentiation (miR-9, miR-30b/c), or with upregulation by inflammatory cytokines (miR-155). Putative tumor-suppressor miRNAs (miR-199a, miR-214, miR-193b, miR-145) were downregulated in PCNSL. There was no overlap of miRNAs dysregulated in PCNSL with those differentially expressed between immunohistologically defined germinal center B cell–like (GCB) and non-GCB types or, apart from miR-9, with miRNAs known to be overexpressed in human brain. We conclude that PCNSL exhibits a distinct pattern of miRNA expression compared with nDLBCL. This argues for the involvement of different molecular mechanisms in the pathogenesis of these two lymphoma types.

Keywords: micro-RNA, Myc, primary CNS lymphoma.

Primary CNS lymphoma (PCNSL) is a rare subtype of extranodal non-Hodgkin’s lymphoma that is confined to the CNS in the absence of systemic disease. Most cases are classified as diffuse large B-cell lymphoma (DLBCL).1 PCNSL is characterized by a poor prognosis compared with systemic lymphoma with similar histology.2 Mechanisms leading to the development of a DLBCL in the CNS, which is normally devoid of a lymphatic system, are still not fully understood.

Recently, the widespread dysregulation of the expression of micro-RNAs (miRNAs) in cancer cells has become evident.3 MiRNAs are small (18 to 24 nucleotides) noncoding RNAs that regulate gene expression by binding to partially complementary target sites in mRNA 3′ untranslated regions, which results in degradation of the target miRNAs or translational repression of the encoded proteins.4 Expression of specific miRNAs has been shown to promote tumorigenesis, whereas other miRNAs exert tumor-suppressor properties.5 MiRNA expression signatures are increasingly used for the classification of cancer and have been associated with prognosis in some cases.6,7

Human B-cell lymphomas including DLBCL overexpress members of the miR-17-92 cluster and also miR-155, both of which are thought to be involved in B-cell lymphomagenesis.8–16 Furthermore, B-cell lymphomas and DLBCL subtypes exhibit different miRNA signatures, reflecting their differentiation stages.17,18

In this work, we investigated the expression of a wide array of miRNA species in PCNSL to search for a differential expression compared with nodal DLBCL (nDLBCL), in an effort to determine pathways potentially involved in PCNSL pathogenesis.
Materials and Methods

Immunohistochemistry

Formalin-fixed and paraffin-embedded (FFPE) biopsy samples from HIV-negative and not otherwise immunosuppressed patients with newly diagnosed PCNSL participating in a controlled therapy trial and from patients with nDLBCL were obtained with informed consent and in accordance with the Declaration of Helsinki. The study was approved by the local ethics committee. Histological diagnosis of DLBCL was confirmed by central pathological review in all cases. Tissue sections were further stained for cluster of designation (CD) 10, B-cell lymphoma 6 (BCL6) protein, and multiple myeloma oncogene 1 (MUM1) (cutoff ≥ 30% positive cells) using standard methods to establish the cell-of-origin (COO) subtype according to the Hans classifier. Immunohistochemical analysis for Myc protein was performed, and the number of positive cells was estimated in 25% steps.

RNA Extraction

For RNA analysis, we selected only samples with a tumor cell content of at least 80%. Under RNase-free conditions, 4 to 6 serial sections (20 μm) were cut from FFPE tissue blocks. Sections were deparaffinized twice with xylene (3 min, 50°C), dehydrated with 100% ethanol, and air dried. RNA was extracted using the RecoverAll Kit (Ambion) following the manufacturer’s instructions (proteinase K digestion for 3 h at 50°C) and eluted in 100 μL of nuclelease-free water (95°C). Remaining DNA was digested using the RNeasy Mini Kit (Qiagen). RNA was finally eluted in 40 μL of RNase-free water and stored at −20°C. A NanoDrop 1000 Spectrophotometer (NanoDrop Technologies) was used to determine RNA concentration.

MiRNA Expression Analysis

For miRNA analysis, 100 ng of total RNA was reverse transcribed in each case using 8 predefined primer pools containing up to 48 multiplex reverse transcription primers each. MiRNA levels were measured by quantitative real-time PCR (qPCR) using commercially available TaqMan low-density arrays (TLDA) together with the 7900HT (high throughput) Fast Real-Time PCR System according to the manufacturer’s recommendations (TaqMan Human MicroRNA Array v1.0; Applied Biosystems). Each TLDA contained specific primer-probe combinations for 365 miRNA species. Each qPCR was analyzed in duplicate. Threshold cycle (Ct) values were determined using Sequence Detection System software (v2.3, Applied Biosystems). Only miRNAs with a Ct value of ≤ 37 in both technical replicates were considered as being present in the sample. To compare the expression of miRNAs not present in a group of samples and putatively expressed in other samples, all Ct values >37 were set to Ct = 38 according to the method of Kubista et al. The TLDA contained 3 internal controls: small nucleolar RNAs relative nucleotide unit (RNU) 44, RNU48, and RNU6B. Because Ct values for RNU48 differed significantly in PCNSL and nDLBCL (P = .005, Mann–Whitney U-test), RNU48 was excluded as an internal control. Data were then independently normalized to RNU44 and to RNU6B, and expression levels were calculated using the 2−ΔΔCt method. Only miRNAs that were differentially expressed after normalization to both internal controls are reported here.

Statistical Analysis

The Mann–Whitney U-test was used to compare the expression of each miRNA between PCNSL and nDLBCL and between germinal center B cell–like (GCB) and non-GCB subtypes. Age distribution in PCNSL and nDLBCL was compared using the t-test, and sex as well as COO subtype were compared using the chi-square test. For bivariate correlations, Pearson’s R was calculated. All tests were two-sided, with P < .05 indicating a significant difference. Statistical analyses were performed using Predictive Analytics Software, version 18. To perform hierarchical clustering of cases and differentially expressed miRNAs, we used Partek Genomics Suite 6.5 beta (Pearson correlation, complete linkage).

Results

Patient Characteristics

MiRNA expression was analyzed in 21 patients: 11 with PCNSL and 10 with nDLBCL. Patient characteristics were balanced between the PCNSL and nDLBCL groups. The median patient age was 65 years (range 53–78) in the PCNSL group and 69 years (63–78) in the nDLBCL group (t-test, P = .39). The male:female ratio was 4:7 in PCNSL and 5:5 in nDLBCL (chi-square test, P = .53), and the non-GCB:GCB subtype ratio was 4:7 in PCNSL and 5:5 in nDLBCL (P = .53). Five patients with nDLBCL had stage III or IV disease, and 5 patients had stage I or II.

MiRNA Expression Levels in PCNSL and DLBCL

Of 365 miRNA species detectable by TLDA, 29% and 33% were present in more than half of the samples of nDLBCL and PCNSL, respectively. The most abundant miRNAs in both lymphoma types were miR-155, miR-92, miR-16, miR-26a, miR-30a-5p, and miR-93 (Fig. 1). We found 18 miRNAs that were differentially expressed between PCNSL and nDLBCL. The expression of 13 miRNAs was significantly higher in PCNSL, whereas 5 miRNAs showed a reduced expression. The expression ratios of specific miRNAs in PCNSL compared with nDLBCL ranged from 13.1-fold overexpression (miR-9) to 3.3-fold reduced expression (miR-145) (Table 1).
In PCNSL we found higher expression of miRNAs belonging to the miR-17-92-cluster (miR-17-5p and miR-20a) and of miR-155, whereas putative tumor-suppressor miRNAs (miR-214, miR-199a, miR-193b, and miR-145) were expressed at lower levels than in nDLBCL (Fig. 2A). Supervised hierarchical clustering employing the differentially expressed miRNAs clearly separated PCNSL and nDLBCL (Fig. 3). An unsupervised analysis did not result in a reliable distinction between PCNSL and DLBCL.

When we compared miRNA expression across all samples according to their immunohistologically defined GCB and non-GCB types, we found 5 miRNAs differentially expressed. None of these miRNAs was among those with differential expression in PCNSL and nDLBCL (Table 1). There was a trend toward higher miR-155 expression in only non-GCB cases ($P = .088$, Fig. 2B).

### Expression of Brain-Enriched MiRNAs

The expression of several miRNAs has been reported to be greatly enriched in the human CNS but not in most other organs. They play an important role in neuronal development and function and therefore are called brain-specific or brain-enriched miRNAs. These include miR-9, miR-124, miR-128, miR-125, and possibly further miRNA species. Only miR-9 exhibited a significantly higher expression in PCNSL than in nDLBCL, whereas the higher expression of miR-124 in PCNSL was of borderline significance ($P = .05$). MiR-128 and miR-125 (Fig. 4) and an additional 19 putatively brain-enriched miRNAs showed similar expression levels in PCNSL and nDLBCL (data not shown). MiR-9 expression in PCNSL did not correlate with tumor cell content ($R = -0.34$, $P = .3$).

### Expression of Clustered MiRNAs

Although formalin fixation is known to have detrimental effects on mRNA quality, it does not affect miRNA expression levels. However, this does not seem to be the case for miRNA expression, as we found similar expression levels in formalin-fixed and fresh-frozen samples. This finding suggests that miRNA expression is less affected by formalin fixation than mRNA expression. However, further studies are needed to confirm this observation.

### Table 1. Micro-RNAs with differential expression in PCNSL and nDLBCL and according to their cell of origin (immunohistochemically determined)

<table>
<thead>
<tr>
<th>Micro-RNA</th>
<th>PCNSL/DLBCL expression ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-9</td>
<td>13.11</td>
<td>.006</td>
</tr>
<tr>
<td>miR-20b</td>
<td>5.97</td>
<td>.017</td>
</tr>
<tr>
<td>miR-155</td>
<td>3.93</td>
<td>.009</td>
</tr>
<tr>
<td>miR-340</td>
<td>3.72</td>
<td>.002</td>
</tr>
<tr>
<td>miR-17-5p</td>
<td>3.60</td>
<td>.029</td>
</tr>
<tr>
<td>miR-148a</td>
<td>3.39</td>
<td>.014</td>
</tr>
<tr>
<td>miR-30b</td>
<td>2.86</td>
<td>.002</td>
</tr>
<tr>
<td>miR-27b</td>
<td>2.65</td>
<td>.020</td>
</tr>
<tr>
<td>miR-26b</td>
<td>2.50</td>
<td>.035</td>
</tr>
<tr>
<td>miR-146b</td>
<td>2.44</td>
<td>.011</td>
</tr>
<tr>
<td>miR-20a</td>
<td>2.40</td>
<td>.035</td>
</tr>
<tr>
<td>miR-30c</td>
<td>2.30</td>
<td>.029</td>
</tr>
<tr>
<td>let-7g</td>
<td>2.10</td>
<td>.007</td>
</tr>
<tr>
<td>miR-199a</td>
<td>0.38</td>
<td>.011</td>
</tr>
<tr>
<td>miR-214</td>
<td>0.36</td>
<td>.006</td>
</tr>
<tr>
<td>miR-432</td>
<td>0.36</td>
<td>.041</td>
</tr>
<tr>
<td>miR-193b</td>
<td>0.32</td>
<td>.005</td>
</tr>
<tr>
<td>miR-145</td>
<td>0.30</td>
<td>.024</td>
</tr>
<tr>
<td>Micro-RNA</td>
<td>GCB/non-GCB expression ratio</td>
<td>P</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>miR-296</td>
<td>5.68</td>
<td>.003</td>
</tr>
<tr>
<td>miR-361</td>
<td>4.38</td>
<td>.013</td>
</tr>
<tr>
<td>miR-301</td>
<td>3.33</td>
<td>.016</td>
</tr>
<tr>
<td>miR-642</td>
<td>2.84</td>
<td>.039</td>
</tr>
<tr>
<td>miR-29c</td>
<td>1.49</td>
<td>.009</td>
</tr>
</tbody>
</table>

*aRatio of median expression levels sorted in descending order.*
analyses, due to the smallness of the molecules. To demonstrate the validity of our miRNA analyses, we tested some of the miRNAs known to be clustered and thus co-regulated for the correlation of their expression in the available lymphoma samples. Members of the miR-17-92 cluster (miR-17-5p, miR-18a, miR-19a, miR-19b, mir-20a, and miR-92) all showed a significant ($P < .05$) correlation of their expression, with $R$-values ranging from 0.473 to 0.953 (median 0.671), irrespective of their differential expression in PCNSL and nDLBCL. The expression of the clustered miRNAs miR-199a and miR-214 was also highly correlated (Online Supplementary Fig. S1).

**Myc Protein Expression**

Several miRNAs (miR-9, miR-17-5p, miR-20a, miR-20b, and miR145) that were differentially expressed in PCNSL are able to contribute to an increased expression of MYC, which is a common oncogenic event in cancer pathogenesis. We therefore looked at Myc protein expression using immunohistochemistry. Tissue sections of 8 nDLBCL and 8 PCNSL were stained for Myc. All cases were Myc positive, exhibiting a heterogeneous staining pattern. In both nDLBCL and PCNSL, the proportion of positive cells was at least 50% (Fig. 5).

**Discussion**

Whether PCNSL and nDLBCL differ in their molecular features and pathogenesis is still uncertain. Gene expression analyses were able to distinguish PCNSL and systemic DLBCL. However, the magnitude of the effect of the CNS background is a matter of ongoing debate. The importance of miRNA dysregulation in cancer etiology, progression, and metastasis has become evident in recent years. Studying miRNAs that regulate gene expression at the posttranscriptional level may...
reveal features specific for PCNSL in contrast to nDLBCL.
and thus improve our understanding of PCNSL pathogenesis. However, data on miRNA expression in PCNSL are very scarce. MiRNAs have recently been utilized as cerebrospinal fluid biomarkers for the detection of PCNSL.31

To date, only one study has reported on the differential
expression of 15 miRNAs (miR-15a, miR-15b, miR-16, miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, miR-21, miR-92, miR-127, miR-155, miR-181a, and miR-221); this study, which examined 9 PCNSL, 19 nDLBCL, 11 extranodal DLBCL, and 11 primary testicular DLBCL, found miR-17-5p to be overexpressed in PCNSL compared with nDLBCL.32

In our study, we examined the expression of a large panel of 365 miRNAs in PCNSL using a qPCR-based array technique. The RNA investigated had been extracted from FFPE samples. The feasibility of this approach had been demonstrated before and is also shown by the strongly correlated expression of clustered miRNAs in our study.18,24,33

Approximately one-third of the analyzed miRNAs were detectable in PCNSL. MiR-155 and miR-92, a member of the miR-17-92 cluster, exhibited the highest expression levels in both nDLBCL and PCNSL, a finding in line with previous reports describing high expression and a possible role in lymphomagenesis for these miRNAs in systemic DLBCL. In mice, an augmented B-cell proliferation and transformation by miR-155 and an accelerated B-cell lymphoma development driven by miR-17-92 have been demonstrated.10,13–15 When we compared miRNA expression in nDLBCL and PCNSL, we found that 18 miRNAs were differentially expressed, 13 were overexpressed, and 5 were downregulated in PCNSL. In addition to a known miR-17-5p overexpression in PCNSL, we found miR-20a, which is also a member of the miR-17-92 polycistron on chromosome 13, and miR-20b, a member of the miR-106a-92 cluster on chromosome X homologous to miR-17-92, to be significantly upregulated in PCNSL. MiR-17-92 is upregulated by the proto-oncogene MYC and thus accelerates MYC-induced lymphoma development.12 Correspondingly, miR-145, which directly represses Myc expression (thus mediating the activity of the tumor suppressor p53) was downregulated in our PCNSL samples.27

Furthermore, we found miR-9 and members of the miR-30 family (miR-30b and miR-30c) to be upregulated in PCNSL. Both are overexpressed in germinal center B cells, in which they downregulate the transcription factor positive regulatory domain (PRDM)-1 (Blimp-1), an essential regulator of plasma cell differentiation.17,24 Blimp-1 is able to repress MYC and thereby facilitates the exit of B cells from the cell cycle and their terminal differentiation into plasma cells.35 Downregulation of Blimp-1 by mutation or epigenetical silencing of the PRDM-1 gene has already been recognized as an oncogenic mechanism in DLBCL and also in one report on PCNSL.16–38 Thus, overexpression of miR-9 and miR-30 may contribute to lymphomagenesis by blocking B-cell terminal differentiation through PRDM-1 silencing. Recently, miR-9 activation by MYC has been demonstrated in breast cancer.26 In PCNSL, the data concerning the role of MYC are few and conflicting.39–42 When we looked for Myc expression in PCNSL by immunohistochemistry, we found a strong expression. We did not perform a direct comparison of the immunohistochemically determined MYC expression in PCNSL and nDLBCL. However, recent gene expression analyses demonstrated a higher expression of MYC mRNA in PCNSL than in nDLBCL.29 Consequently, an activation of the MYC pathway in PCNSL seems likely. Nonetheless, further targets of the dysregulated miRNAs are known and might be relevant in PCNSL. Members of the miR-17-92 cluster downregulate a number of proapoptotic and tumor-suppressive factors, such as CDKN1A/p21, phosphatase and tensin homolog (PTEN), E2F1, and Bim.8,9,11 For instance, downregulation of Bim leads to overexpression of BCL2, which is a prominent feature in PCNSL.41,43 Other miRNAs that were downregulated in PCNSL, namely miR-199a, miR-214, and miR-193b, have been demonstrated to act as tumor suppressors via downregulation of certain oncogenes in different solid cancers.44–47

Another striking finding of our study was the significant overexpression of miR-155 in PCNSL. This is in contrast to findings of the previously published miRNA study on PCNSL and is possibly explained by a different sample preparation, varying RNA quality, and low sample size in both our study and theirs.32 MiR-155 possesses an oncogenic potential, as demonstrated in a lymphoma model in mice.13 Physiologically it is expressed in activated B and T cells and monocytes and controls the germinal center reaction by regulating the production of certain cytokines such as tumor necrosis factor (TNF).48,49
Normal CNS lacks the formation of germinal centers, but under certain circumstances, specifically in inflammatory CNS diseases, ectopic germinal centers have been observed within the meninges.\(^{50,31}\) An association of inflammatory processes and lymphoma development in PCNSL pathogenesis has been hypothesized. Mir-155 has been reported to be upregulated by immune stimuli such as TNF-\(\alpha\) and on the other hand can render nDLBCL resistant to growth-inhibitory cytokines such as transforming growth factor-\(\beta\).\(^{52-55}\) However, conclusive data regarding the possible role of inflammation in PCNSL pathogenesis are still lacking, and further studies are necessary.

An overexpression of miR-155, as well as the differential expression of a number of further miRNAs in immunohistologically defined non-GCB type DLBCL, has been reported by some authors.\(^{14,53,36}\) According to gene expression data, PCNSL are heterogeneous, in particular regarding their COO type. Notwithstanding the limitations of the Hans classifier, which furthermore has not been tested in PCNSL, we used this immunohistological classification to avoid a potential bias introduced by a predominance of one COO type in the PCNSL or nDLBCL samples examined. Still, this and other molecular signatures might have superimposed the specific differences between PCNSL and nDLBCL detectable by statistical approaches.

We cannot exclude the possibility that our miRNA expression data for PCNSL may in part be derived from the CNS background. As noted, miR-9, which was most strongly overexpressed in PCNSL, belongs to the group of brain-specific or brain-enriched miRNAs.\(^{22,23,57}\) However, miR-9 is also strongly involved in B-cell maturation, and together with miR-124 was the only brain-enriched miRNA highly expressed in PCNSL in our study, whereas other members of this group exhibited similar expression levels in nDLBCL and PCNSL.

In summary, in this study we were able to detect a differential miRNA expression pattern in PCNSL. Dysregulation of specific miRNAs leading to, for instance, an activation of the MYC pathway or to a blockade of germinal center exit in B cells by Blimp-1 downregulation might play a role in PCNSL pathogenesis. Further studies are warranted to better describe the targets of the identified miRNAs.

Supplementary Material
Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

Acknowledgments
None.

Conflict of interest statement. None declared.

Funding
No external funding for this study.

References

