MIRNAs and their target genes in B cell lymphomas
Tan, Lu Ping

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miRNA analysis in B-cell chronic lymphocytic leukaemia: proliferation centres characterized by low miR-150 and high BIC/miR-155 expression

Miao Wang, Lu Ping Tan, Menno K Dijkstra, Kirsten van Lom, Jan-Lukas Robertus, Geert Harms, Tjasso Blokzijl, Klaas Kooistra, Mars B van t’Veer, Stefano Rosati, Lydia Visser, Mojca Jongen-Lavrencic, Philip M Kluin and Anke van den Berg.

Abstract
Several miRNAs have been reported to be associated with immunoglobulin heavy chain (IgH) mutation and ZAP-70 expression status in blood samples of B-cell chronic lymphocytic leukaemia/small lymphocytic lymphoma (B-CLL/SLL). In the bone marrow and lymphoid tissues, proliferation centres (PCs) represent an important site of activation and proliferation of the neoplastic cells, suggesting that these tissues better reflect the biology of CLL than circulating blood cells. We collected 33 lymph nodes and 37 blood CLL samples and analysed IgH mutation status and ZAP-70 expression status. Expression of 15 miRNAs was analysed by qRT-PCR and RNA-ISH. Sixty-three per cent of the lymph node cases contained mutated IgH genes and 49% of the lymph node cases were ZAP-70-positive, and a significant correlation was observed between ZAP-70 expression and IgH mutation status. Of the blood CLL samples, 49% contained mutated IgH sequences. The miRNA expression pattern in CLL lymph node and blood samples was very similar. Three of 15 miRNAs (miR-16, miR-21, and miR-150) showed a high expression level in both blood and lymph node samples. No difference was observed between ZAP-70-positive or -negative and between IgH-mutated or unmutated cases. No correlation was found between miR-15a and miR-16 expression levels and 13q14 deletion in the blood CLL samples. RNA in situ hybridization (ISH) revealed strong homogeneous staining of miR-150 in the tumour cells outside the PCs. In reverse BIC/pri-miR-155 expression was observed mainly in individual cells including prolymphocytes of the PCs. This reciprocal pattern likely reflects the different functions and targets of miR-150 and miR-155.
Chapter 3

Introduction

B-cell chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) is characterized by the accumulation of mature CD5+, CD19+, CD23+ B-lymphocytes. From a prognostic point of view, CLL represents a heterogeneous disorder. Indolent versus aggressive forms of the disease can be discriminated by a magnitude of clinical, morphological, kinetic, immunophenotypic, cytogenetic, and molecular features [1,2]. In 1999, two reports [3,4] showed that CLL cases with unmutated IgH genes, ie homology of ≥98% with germline sequences, are associated with a more aggressive form. Later on, two reports [5,6] showed that CLL cases with unmutated IgH genes have a distinct gene expression profile with active signaling of the B-cell receptor (BCR) and are frequently ZAP-70-positive.

In CLL, the so-called proliferation centres (PCs) can be found at the tissue level. These structures contain a slightly increased number of T-cells and in a few cases, also some dendritic cells. The neoplastic cells in these structures are more activated than the surrounding neoplastic cells and circulating cells, reflected by higher expression of a number of antigens including immunoglobulin, CD38, Ki67/MIB1, CD23, and MUM1/IRF4 [7,8]. One of the most common genomic aberrations in CLL is deletion of the 13q14 region. According to Calin et al [9], the miR-15a and miR-16-1 polycistron is the most likely target in this genomic region. A homologous miRNA cluster is located at 3q26.1, which contains miR-15b and miR-16-2. Based on these initial observations, several groups have studied these and other miRNAs in B-CLL, other lymphomas, and normal B-cells, and it has become clear that miRNAs play a crucial role in lymphomagenesis. miR-15, miR-16, miR-21, miR-92, miR-101, miR-150, miR-155, and miR-181a are all highly expressed in B-CLL [10–12]. miR-92 is a member of the miR-17-92 polycistron (miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b, miR-92a), which is located in the 13q31 region that frequently shows copy number gains or even amplifications in various NHL subtypes [13,14].

We have investigated miRNA expression in lymph node and blood CLL by qRT-PCR followed by RNA in situ hybridization (ISH) and show that miRNA expression levels are not correlated with ZAP-70, IgH mutation, and 13q14 deletion status.
**Materials and methods**

**CLL samples**

Formalin-fixed and paraffin-embedded tissue samples of 33 CLL cases were obtained from patients diagnosed with CLL in the Department of Pathology and Laboratory Medicine, University Medicine Centre Groningen. All protocols for obtaining and studying human tissues and cells were approved by the institution’s review board for human subject research. CLL diagnosis was based on clinical manifestation, blood leukocyte and lymphocyte counts, morphology and immunophenotyping in accordance with the WHO classification guidelines [15]. In 31 cases, a leukaemic picture was present in addition to the lymphoma component. Two cases presented as lymphoma without a leukaemic picture in the blood. Thirty seven CLL blood samples were collected from the Department of Hematology, Erasmus MC, Rotterdam. These cases have previously been analysed for IgH mutation and 13q14 deletion status [16] (see Supplementary Tables 2 and 3).

**Immunohistochemistry of ZAP-70**

ZAP-70 immunohistochemistry was performed as previously described for the lymph node cases [8]. Immunostaining was performed using anti-ZAP-70 antibody (clone 2F3.2; Upstate Biotechnology) at a dilution of 1:100. The tumour was considered strongly positive when the staining intensity was similar to that observed in T-cells, weakly positive when the staining intensity of the tumour cells was much less than the infiltrating T-cells, and negative when no staining was observed in the tumour cells.

**Determination of IgH somatic hypermutations**

DNA was extracted from lymph node specimens using standard procedures and IgH amplification was carried out in duplicate, as described previously [17,18], including negative controls. PCR products were analyzed on MegaBase (Amersham Bioscience, Midland, Ontario, Canada). The PCR products containing one rearranged allele were spin column-purified (Qiagen, Valencia, CA, USA) and sequenced using dye terminator chemistry (Big Dye Kit; Applied Biosystems, Foster City, CA, USA).
City, CA, USA) on a genetic analyser model 377 (Applied Biosystems). If two peaks were observed, the PCR products were cloned and multiple clones were sequenced. Consensus IgH sequences were compared with the germline sequence using Ig-BLAST (http://www.ncbi.nlm.nih.gov/igblast/). The consensus sequence with a functional open reading frame was used to determine the mutation status. Cases with homology of ≥98% with germline sequences were considered as unmutated, and cases with homologies <98% as mutated.

**TaqMan qRT-PCR miRNA analysis**

RNA was extracted from the lymph nodes as reported previously by Specht *et al* [19] and from the blood samples using RNABee (Campro Scientific, Veenendaal, The Netherlands). Turbo DNase treatment and qRT-PCR for mature miRNAs were performed as described by the manufacturer (Ambion, Foster City, CA, USA). U6 was used for normalization (ΔCt = Ct_miRNA − Ct_U6) and to check the quality of the samples, ie only cases with a cycle threshold (Ct) value lower than 30 were used, resulting in a total of 28 cases. The primers used were as follows: U6 forward primer: 5’-tggaacgatacagagaagattagca-3’ and reverse primer 5’-aaaatatggaacgcttcacgaatt-3’. Fifteen miRNAs including the chromosome 3 and 13 miR-15-16 clusters (miR-15a, miR-15b, miR-16-1, and miR16-2), all members of the miR-17-92 polycistron, and five miRNAs known to be highly expressed in B-CLL (miR-181a, miR-155, miR-21, miR-101, and miR-150) were analysed by qRT-PCR. Relative expression levels were determined by using the formula $2^{-\Delta Ct}$.

**RNA in situ hybridization (ISH)**

RNA-ISH was performed as described previously [20,21]. miRNA staining was performed using an LNA probe antisense to miR-16, miR-92, miR-155, miR-21, and miR-150 (Exiqon, Denmark) in eight CLL cases, according to the manufacturer’s protocol. BIC is the primary miRNA (pri-miR-155) transcript that can be processed to mature miR-155. DIG-labelled BIC RNA probes were made with the DIG RNA
miRNA profile of CLL

Labeling Kit (Sp6/T7) (Roche, Mannheim, Germany) to detect the full-length primary BIC transcript.

Statistical analysis
The correlation between ZAP-70 and IgH mutational status was analysed with Pearson’s chi-square test. The correlation of miRNA expression levels with ZAP-70 or IgH mutational status was analysed with the unpaired t-test. All \( p \) values were two-sided and \( p < 0.05 \) was considered to be significant. SPSS 14.0 software was used for the analysis.

Results
ZAP-70 expression is associated with IgH mutation status
IgH analysis was successful for 27 out of 33 CLL lymph node samples. Ten cases (37%) contained unmutated IgH genes and 17 cases (63%) carried mutated IgH genes (see Supplementary Table 1). Of the blood CLL samples, 18 cases (49%) contained mutated IgH sequences. In normal tonsils, ZAP-70 stained intensively positive in the T-cells located in the inter-follicular areas, whereas only a few positive cells were observed in the follicles. In ZAP-70-positive CLL paraffin tissues, all tumour cells, or at least more than 70%, stained positive, whereas in ZAP-70-negative cases, no recognizable tumour cells stained positive. In total, 16 (49%) of the CLL lymph node cases were ZAP-70-positive and 17 (51%) of the cases were ZAP-70-negative (see Supplementary Table 1). Combination of the IgH mutation status and the ZAP-70 staining in the lymph node revealed that ten of the 13 ZAP-70-positive cases carried unmutated and three cases carried mutated IgH genes. All 14 ZAP-70-negative cases carried mutated IgH genes. In 11% of the CLL cases (3/27), ZAP-70 status was discordant with IgH mutational status (see Supplementary Table 1). Overall, there was a significant correlation between the IgH mutation status and the ZAP-70 staining results in CLL lymph node paraffin tissues (\( p < 0.01 \)).
Figure 1. Schematic representation of the miR-17-92 and the miR-15–16 clusters and miRNA profile in CLL cases. (A) Schematic representation of the miR-17-92 cluster located on chromosomal region 13q31. Schematic representation of the two miR-15–16 clusters located on chromosomal regions 13q14.3 and 3q26.1. The sequences of the mature miRNAs miR-15a and miR-15b differ at four positions, whereas the sequences for miR-16-1 and miR-16-2 are identical. (B) CLL cases from lymph node samples show the most abundant expression of miR-150, and intermediate expression levels for miR-16, miR-21, miR-92, and miR-155. (C) CLL cases from blood show the most abundant expression of miR-150, and intermediate expression levels for miR-16, miR-21, miR-19b, and miR-20a. The difference in the relative expression levels between the blood and lymph node samples (see scale on the x-axis) is caused by the poorer RNA quality in the paraffin-embedded lymph node samples.

Similar miRNA expression pattern in CLL lymph node and blood samples
Analysis of 15 miRNAs by qRT-PCR revealed the highest expression level for miR-150 and an intermediate expression level for miR-16 and miR-21 in both lymph node and blood CLL. Eight microRNAs (miR-15a, 15b, 17-3p, 17-5p, 18a, 19a, 181a, and 101) showed very low expression in all samples. In lymph node samples, an intermediate expression level was also observed for miR-155 and miR-92a, whereas a higher level of expression of miR-19b and miR20a was observed in the blood samples (Figure 1). For miR-15, the expression level of the chromosome 3-derived miR-15b was higher than that of the chromosome 13-derived miR-15a. The
expression level of miR-16 was higher than those of miR-15a and miR-15b (Figure 1B). However, based on the identical sequences of miR-16-1 and miR-16-2, it is not possible to discriminate between the chromosome 3 and 13 copies.

No correlation of the expression levels of the 15 miRNAs with IgH mutation or ZAP-70 status

We did not find a significant correlation between the expression level of any of these 15 miRNAs and IgH mutation or ZAP-70 status (Figures 2 and 3). A trend of a higher expression level of miR-16 ($p = 0.06$) was observed in unmutated CLL blood samples, but not in lymph node samples. Although not significant, miR-150 expression levels were higher in CLL cases carrying mutated IgH sequences.

No correlation of the expression levels of miR-15a and miR-16 with 13q14 deletion status
The 13q14 deletion status was determined in the 37 blood samples by using three FISH probes (see Supplementary Table 3). Comparison of the expression levels of miR-15a and miR-16 in cases without and with deletions revealed no differences in the expression levels for any of the three probes used for the FISH analysis (Figure 4). In general, all the CLL cases showed a consistent low expression level for these two miRNAs.

![Figure 4. Correlation of miR-15a and miR-16 levels with 13q14 deletion status.](image)

(A, B) Correlation of the miR-15a and miR-16 levels with the 13q14 deletion status of D13SPRB-1 probe detection. No differences were observed between 13q14 undeleted and deleted cases. (C, D) Correlation of the miR-15a and miR-16 levels with the 13q14 deletion status of D13S319 and D13S25 probe detection. No differences were observed between 13q14 undeleted and deleted cases for the miRNAs. undel = no deletion; del = deletion.
miR-150 and BIC/pri-miR-155 demonstrated an inverse expression pattern in PCs and surrounding cells

ISH analysis of miR-150 and BIC/pri-miR-155 demonstrated a specific expression pattern for PCs and surrounding tumour cells. For miR-150, relatively strong and homogeneous staining was observed for the neoplastic cells outside the PCs, whereas almost no cells within the PCs stained positive. This pattern was observed in all five cases that could be analysed (in three cases, the staining failed; Figures 5B and 5C). For BIC/pri-miR-155, we observed an almost reciprocal pattern, most positive cells being localized within the PCs in seven of eight cases analysed (one failure). Some of the positive cells could be easily identified as prolymphocytes, indicating that these are neoplastic CLL cells (Figure 5F). The smaller cells that were positive might represent small CLL cells or T-cells that also accumulate in the PCs. Double-staining procedures...
to identify the nature of these smaller cells failed (data not shown). The number of BIC/pri-miR-155-positive cells varied from patient to patient, which was consistent with the variance in the size and number of PCs in the individual cases (Figures 5D–5F). The staining of miR-16, miR-21, miR-92, and miR-155 failed, probably due to the lower expression levels.

**Discussion**

ZAP-70 and IgH mutation status both represent good prognostic markers and more recent reports also indicate that miRNAs might represent valuable prognostic tools. Most studies have focused on the analysis of circulating blood cells, whereas lymph nodes might better reflect the activation status and function of the neoplastic cells than circulating cells. In this study, we analysed blood and lymph node CLL. Both the percentage of ZAP-70-positive cases (49%) and the percentage of IgH-mutated cases (lymph node 63%; blood 49%) and the correlation are in line with the general findings reported by others [5,6,22–25]. Discordance between ZAP-70 and IgH mutation status was observed in only three of the 27 cases (11%). These cases were ZAP-70-positive but contained mutated IgH genes, including one case with a VH3-21 germline sequence. CLL cases with both mutated and unmutated VH3-21 germline sequences are usually ZAP-70-positive and are characterized by a poor prognosis independent of mutation status [26–29].

Several studies [10,11,30] showed that miRNA expression is deregulated in CLL blood samples and that this may have clinical (prognostic) significance. Calin et al [10,11] reported miRNA profiles in CLL blood samples using microarrays and found altered expression in comparison to normal CD5+ B-cells. Fulci et al [12] showed that miR-16, miR-21, miR-101, miR-150, and miR-155 were the most abundantly expressed miRNAs in CLL blood samples by a miRNA cloning approach. Four of these miRNAs were also expressed at a higher level in CLL compared with normal CD19+ B-cells and normal CD5+, CD19+ cord blood cells as determined by qRT-PCR. Using qRT-PCR, we found similar high expression of the same miRNAs in our cases, with miR-150 being the most abundant in all samples. Our data are quite
miRNA profile of CLL

consistent with those of Fulci et al [12], who also reported high expression levels of the same miRNAs. Moreover, we observed a very similar miRNA expression pattern in CLL lymph node and blood samples, suggesting that the tumour cells in both presentation forms are comparable with respect to miRNA regulation. The difference between our data and those obtained by Fulci et al [12], compared with Calin et al [10, 11, 30], might be caused by differences in the technical approach. Using microarrays, the focus is on differential expression patterns and not on differences in absolute expression levels, whereas cloning and qRT-PCR also allow comparison of the relative abundance of individual miRNAs. At this moment, qRT-PCR specific for mature miRNAs seems to provide more accurate results and is considered to be the gold standard [31].

Of the seven miR-17-92 cluster members, miR-92 was most abundant in lymph node, whereas miR-20a and miR-19b were the most abundant miRNAs in blood. The other cluster members were expressed only at a very low level. It can be speculated that high miR-19b and miR-92 levels are derived from the homologous miR-106a–miR-92 cluster located on Xq26.2. However, this does not explain the differences between lymph node and blood CLL or the higher expression level of miR-20a in the blood samples. Most likely other factors related to processing or miRNA stability contribute to the high level of miR-92 or miR-19b and miR-20 in CLL.

At this moment, it is unclear which genes are targeted by these miRNAs and how overexpression of these miRNAs in CLL contributes to lymphomagenesis. Of the miR-15–16 clusters present on chromosomes 3 and 13, miR-16-1 and miR-16-2 are identical and cannot be detected separately, whereas miR-15a and miR-15b differ in four nucleotides (Figure 1). Deletion of chromosome band 13q14 is the most frequent aberration found in CLL and is associated with reduced levels of miR-15a and miR-16 [9]. In our 37 blood samples, we found 13q14 deletions in about half of the cases. Cytogenetic abnormalities of chromosome 3 carrying the second miR-15–16 cluster are very rare in B-CLL. In our CLL samples, miR-16 levels were higher than miR-15a and miR-15b levels, which might suggest that a major part of miR-16 expression is derived from the primary transcript from chromosome 3. Expression of
the chromosome 3 miRNA cluster might also explain the lack of correlation between
the CLL cases with and without 13q14 deletions.

Another observation made by Calin et al and Fulci et al is the significant correlation
of some miRNAs with ZAP-70 status [10–12]. In the first study [10] miR-15a, miR-21,
and miR-92 were among the miRNAs that could discriminate between the two main
CLL clusters. In the second study [11], miR-155 was differentially expressed
between ZAP-70-positive and ZAP-70-negative cases. miR-15a and miR-16 were
also differentially expressed, but miR-21 and miR-92 were not. In contrast to these
findings, we did not find any correlation between the expression levels of the 15
miRNAs and ZAP-70. Some of these discrepancies might be related to the poorer
quality of the first generation of miRNA microarrays, which were not optimized for
differences in the hybridization temperatures for each individual miRNA and were
not specific for the mature miRNA sequences. Fulci et al [12] reported that miR-150
was differentially expressed between IgH-mutated and IgH-unmutated CLL cases.
Although we saw the same trend in the CLL samples, the difference was not
significant in our study.

Our RNA-ISH indicated that miR-150 was expressed in all tumour cells except the
PCs, whereas BIC /pri-miR-155 was expressed exclusively in the PCs. These data
appear to be consistent with the qRT-PCR data of the CLL lymph node samples that
show a much higher expression level of miR-150 compared with miR-155. These
results suggest that BIC /pri-miR-155 and miR-150 might play a role in the PCs of
CLL. In lymph nodes and the bone marrow, PCs are the hallmark of this
lymphoproliferative disorder and the tumour cells in PCs show a distinct antigen
expression profile (CD38, Ki67/MIB1, CD23, and MUM1/IRF4 [32,33]). In CLL blood
samples, we and Fulci et al [12] also demonstrated a very high level of miR-150
compared with miR-155. The relative abundance of miR-155 compared with miR-
150 is much higher in the lymph node CLL samples. Both BIC /pri-miR-155 and the
mature form of miR-155 are highly expressed in Hodgkin, primary mediastinal, and
diffuse large B-cell lymphomas [21,34–36], which supports a role in
lymphomagenesis. In a transgenic mice model with overexpression of miR-155,
Costinean et al [37] demonstrated a pre-B-cell proliferation followed by a polyclonal
miRNA profile of CLL

B-cell malignancy. In the transgenic miR-150 mice model, miR-150 controls B-cell differentiation by targeting the c-myb transcription factor [38]. Both miR-150 and miR-155 play a main role in regulation of the immune response, as demonstrated by genetic deletion and transgenic approaches [38–40]. To our knowledge, we have shown for the first time the specific miR-150 and BIC /pri-miR-155 expression pattern in CLL. Unfortunately, we could not obtain a good RNA ISH signal for the mature miR-155 in CLL lymph node cases. This probably reflects the hybridization kinetics of the probe, since we also could not obtain a very strong hybridization signal for miR-155 in the tumour cells of Hodgkin lymphoma. An alternative explanation for the lack of miR-155 signal might be that BIC /pri-miR-155 is not processed properly, but this lack of processing has until now only been demonstrated in Burkitt lymphoma, which also lacks endogenous expression of BIC [41,42]. The low expression of miR-150 in combination with the high expression of pri-miR-155 in the PCs needs to be further studied to determine its biological relevance in CLL.

In summary, we observed no difference in the expression levels of our selection of 15 miRNAs based on ZAP-70 and IgH mutation status. miR-150 is weakly expressed in the PCs and strongly in the surrounding cells, whereas BIC /pri-miR-155 expression is restricted to the PCs of CLL lymph node cases. These findings suggest a role for miR-150 and BIC /pri-miR-155 in the regulation of proliferation and/or activation of CLL cells.

All supplementary data is available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2333.html

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