MicroRNA as biomarkers and regulators in B-cell chronic lymphocytic leukemia

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Early cancer detection and disease stratification or classification are critical to successful treatment. Accessible, reliable, and informative cancer biomarkers can be medically valuable and can provide some relevant insights into cancer biology. Recent studies have suggested improvements in detecting malignancies by the use of specific extracellular microRNAs (miRNAs) in plasma. In chronic lymphocytic leukemia (CLL), an incurable hematologic disorder, sensitive, early, and noninvasive diagnosis and better disease classification would be very useful for more effective therapies. We show here that circulating miRNAs can be sensitive biomarkers for CLL, because certain extracellular miRNAs are abundant in CLL plasma and better correlates with survival than from healthy controls and from patients affected by other hematologic malignancies. The levels of several of these circulating miRNAs also displayed significant differences between zeta-associated protein 70 (ZAP-70)+ and ZAP-70− CLL. We also determined that the level of circulating miR-20a correlates reliably with diagnosis-to-treatment time. Network analysis of our data, suggests a regulatory network associated with BCL2 and ZAP-70 expression in CLL. This hypothesis suggests the possibility of using the levels of specific miRNAs in plasma to detect CLL and to determine the ZAP-70 status.

Chronic lymphocytic leukemia (CLL) is the most common hematologic malignancy affecting adults in Western countries, even though the true incidence remains uncertain (1). Like many other cancers, the diagnosis of the disease is often delayed due to the lack of symptoms in the early stages. The abnormal expression of certain T-cell markers by CLL B cells, namely CD5, CD184, and ZAP-70, have been helpful in stratifying the disease (2). For example, overall survival is significantly better for ZAP-70+ than for ZAP-70− cases. ZAP-70 is primarily a T- and NK-cell marker in differentiated cells and also plays a role in the transition of pro-B to pre-B cells in the bone marrow (3). Thus, ZAP-70+ CLL seems to represent less differentiated cell populations and has a more aggressive cancer phenotype. Although CLL remains an incurable disorder, early stage detection and treatment can control disease progression, whereas late stage patients are often unresponsive to various treatments (4). Even though CLL often presents with a heterogeneous pathology in the clinic, most patients receive a few standardized treatments, primarily on the basis of limited clinical parameters such as Rai or Binet staging, which classify CLL patients on the basis of the spreading of the disease and its cytogenetic characterization, underlining the need for more informative diagnostic markers with better clinical significance (5, 6).

Since the recent reports of circulating, extracellular microRNAs (miRNAs) in plasma, they have become an attractive source of new nucleic acid-based biomarkers (7). MicroRNAs belong to the class of small noncoding RNA molecules (∼20 nucleotides) and affect biological functions either by posttranscriptional silencing or stimulating transcript degradation. It is estimated that the expression of 20–30% of protein-coding genes may be affected by either one or multiple miRNAs (see ref. 10 for example). The levels of miRNAs are precisely controlled in the cells to assure proper cellular function and differentiation (8); aberrant expression of miRNAs are commonly observed in pathological processes, including oncogenesis (9). One commonly observed chromosomal aberrations in CLL is the deletion of chromosomal 13q14.3, a region containing miR-15a and miR-16, which suggests, but does not prove, the involvement of miRNAs in the pathogenesis of CLL (10). In addition, an aberrant cellular miRNA expression profile in CLL B cells has been described and the changes correlate well with prognostic factors including ZAP-70 expression status and IgVH mutations in CLL patients (11). Recent studies also demonstrated the decrease of miR-29c and miR-223 levels in cells during the progression of the disease (12).

Prior studies have reported the presence of tumor-derived, low molecular weight RNAs (miRNAs and other noncoding RNAs) in plasma of patients with solid tumors (13–15). In the present study, our aim was to explore the possibility of using the changes of extracellular miRNA spectra in CLL plasma samples to provide more accurate assessment of the disease and improve the molecular classification of CLL. On the basis of our findings, specific plasma miRNA signatures are associated with CLL and these miRNAs could constitute new and informative biomarkers in CLL diagnosis and disease stratification.

Results
We investigated the changes of plasma miRNA spectrum from a cohort of CLL patient plasma samples (SI Appendix, Table S1 summarizes clinical information on cohort). The CLL prognosis markers, ZAP-70 expression level and IgVH mutation status from the corresponding B cells were also determined. To identify miRNA with potential clinical applications, we initially profiled miRNA spectra from nine pooled samples including pooled healthy control, six pooled CLL samples based on disease stages and ZAP-70 expression status, a pooled multiple myeloma (MM) plasma, and a pooled sample with hairy cell leukemia (HCL). The miRNAs with altered expression levels in each set were then verified with individual samples in the cohort, which contains 31 patient and 17 healthy control samples.

Significant Number of miRNAs Are Present in Plasmas of Patients with B-Cell–Related Malignancies. Using unsupervised hierarchical clustering, the samples were grouped into three major groups, the ZAP-70+ samples, the ZAP-70− samples, and a group containing normal, MM, and HCL samples based on the results of plasma miRNA profile (SI Appendix, Fig. S1). Similar to prior findings in plasma from patients with solid tumors, a higher number of detectable circulating miRNAs was found in CLL plasma samples (average n = 296) compared with normal, control plasma (n = 230), a 28% increase. A similar trend was also observed in other hematologic malignancy samples tested: an MM plasma sample had 256 detectable miRNAs, an 11% increase, and an HCL sample

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Table 1. Discriminating CLL from healthy controls (P < 0.01) and other hematologic malignancies with circulating miRNA

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Mean fold change ± SEM</th>
<th>AUC value</th>
<th>Mean fold change ± SEM</th>
<th>P value</th>
<th>Mean fold change ± SEM</th>
<th>P value</th>
<th>Mean fold change ± SEM</th>
<th>P value</th>
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<tr>
<td>Discovery set</td>
<td>B-CLL (n = 41)</td>
<td>Controls (n = 8)</td>
<td>MM (n = 7)</td>
<td>HCL (n = 4)</td>
<td></td>
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<tr>
<td>miR-150</td>
<td>61.83 ± 16.70</td>
<td>7.80E-01</td>
<td>1.09 ± 0.39</td>
<td>1.10E-03</td>
<td>0.43 ± 0.12</td>
<td>1.00E-03</td>
<td>1.63 ± 0.14</td>
<td>1.20E-03</td>
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<td>miR-150*</td>
<td>29.07 ± 7.20</td>
<td>8.10E-01</td>
<td>1.00 ± 0.03</td>
<td>2.30E-03</td>
<td>1.43 ± 0.26</td>
<td>2.70E-03</td>
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<td>miR-29a</td>
<td>24.27 ± 6.79</td>
<td>9.20E-01</td>
<td>1.07 ± 0.15</td>
<td>1.50E-03</td>
<td>0.69 ± 0.12</td>
<td>1.30E-03</td>
<td>1.12 ± 0.58</td>
<td>1.60E-03</td>
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<td>miR-135a*</td>
<td>20.81 ± 5.62</td>
<td>8.40E-01</td>
<td>1.17 ± 0.26</td>
<td>1.60E-03</td>
<td>1.24 ± 0.24</td>
<td>1.60E-03</td>
<td>1.27 ± 0.25</td>
<td>1.70E-03</td>
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<td>miR-195</td>
<td>8.77 ± 1.09</td>
<td>9.50E-01</td>
<td>1.34 ± 0.43</td>
<td>8.90E-08</td>
<td>1.62 ± 0.42</td>
<td>2.00E-07</td>
<td>1.25 ± 0.37</td>
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<td>miR-21</td>
<td>8.76 ± 1.81</td>
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<td>miR-93</td>
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<td>2.68 ± 0.62</td>
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<td>miR-486-5p</td>
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<td>3.38 ± 1.37</td>
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<td>miR-483-5p</td>
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<td>7.30E-01</td>
<td>1.18 ± 0.26</td>
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<td>0.00</td>
<td>NA</td>
<td>1.26 ± 0.15</td>
<td>3.90E-03</td>
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<td>miR-222</td>
<td>5.53 ± 0.86</td>
<td>9.10E-01</td>
<td>1.21 ± 0.25</td>
<td>1.50E-05</td>
<td>1.12 ± 0.30</td>
<td>1.50E-05</td>
<td>1.48 ± 0.74</td>
<td>3.10E-03</td>
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<td>miR-15a</td>
<td>3.03 ± 0.44</td>
<td>6.70E-01</td>
<td>1.08 ± 0.16</td>
<td>2.30E-04</td>
<td>1.07 ± 0.18</td>
<td>3.30E-04</td>
<td>2.20 ± 0.72</td>
<td>3.67E-01</td>
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</table>

[Ct values were normalized by using synthetic spiked-in Caenorhabditis elegans miRNAs and fold changes were calculated relative to the average expression in plasma of healthy controls by using the 2^∆∆Ct method. AUC values were obtained from the ROC curves assessing the predictive power of individual miRNAs. P values were calculated by two-sided t test for each group relative to CLL patients.]

had 260 detectable miRNA species, a 13% increase compared with normal. The plasma samples from ZAP-70⁺ CLL patients had a higher number of detectable miRNAs than the ZAP-70⁻ samples: 335 detectable miRNA species in ZAP-70⁺ vs. 256 detectable miRNA species in ZAP-70⁻ samples (SI Appendix, Fig. S2).

Examining the list of the 20 most abundant miRNAs in all our plasma samples, a significant number of miRNA species including miR-150, miR-19b, and miR-92a, miR-223, miR-320, miR-484, and miR-17 were highly abundant in all of the samples (SI Appendix, Table S2). miR-223 was the most abundant miRNA species in normal human plasma as well as plasma from HCL and MM patients; however, in ZAP-70⁺ CLL samples the miR-150 was the most abundant.

Levels of Specific Circulating miRNAs Can Be Used to Identify Different Hematological Malignancies. From the global miRNA
profiling results with pooled plasma samples, several miRNA species showed distinct profiles between CLL and HCL or MM samples (SI Appendix, Fig. S3A and B). For example, the levels of miR-181a and miR-708 were lower in CLL but higher in HCL and showed no significant difference compared with normal levels in MM (SI Appendix, Fig. S3A), whereas miR-34a and miR-564 were lower in MM but higher in CLL with no significant changes in HCL compared with normal levels (SI Appendix, Fig. S3B). Among the CLL samples, there were a number of miRNA species that showed differences between the ZAP-70+ and ZAP-70− samples (Table S3). For instance, the levels of miR-205, miR-29a, and miR-652 are higher in ZAP-70+ samples compared with ZAP-70− plasma samples, whereas miR-19b and miR-144* were higher in ZAP-70− samples. A number of miRNAs also showed progressive changes along with the severity of the disease; for example, miR-30c concentration in plasma continues to decrease as disease progresses, whereas the miR-363 level continues to increase as disease progresses, regardless of their ZAP-70 expression status (SI Appendix, Fig. S4). Although not all B-cell–derived malignancies were tested, these miRNAs have the potential to be used to distinguish different types of B-cell malignancies (like HCL and MM) or to classify CLL patients by stage, once they have been validated on independent cohorts.

To assess the potential of using miRNAs as CLL biomarkers, we chose 27 miRNAs that showed changes to be studied in individual plasma samples, including normal, 41 CLL, 7 MM, and 106 MM plasma samples. Among the selected miRNAs, 14 of the 27 could clearly discriminate CLL samples from the three other groups (normal, MM, and HCL) with high confidence (P < 0.01, listed in Table 1). The overall distribution of levels of four of these miRNAs (miR-29a, miR-150*, miR-195, and miR-222) between CLL and normal are shown as examples in Fig. 1. miRNAs that showed the most difference between CLL and normal samples in the discovery cohort (miR-150, -150*, -29a, and -135a*) were also observed and confirmed with a second independent validation cohort (also in Table 1), which consisted of 31 CLL patients and 17 healthy controls (SI Appendix, Table S1 for patient information).

The levels of these selected 27 miRNAs were also determined in the corresponding B cells and plasma samples of each patient. Using supervised hierarchical clustering, these miRNAs clearly separated the cells and plasma (SI Appendix, Fig. S5) from each other. The profiles are distinct and clearly different. The cells, in general, had higher levels of miRNAs of most corresponding species in the plasma, except for miR-135a−, miR-451, and miR-486–5p (SI Appendix, Fig. S5). The cellular miRNA spectra further grouped the patients into two subgroups (groups 1 and 2) and three subgroups (groups 1, 3, and 4). Group 4 had all of the controls and most of the ZAP-70− samples, whereas the other two groups were mixed with different stages of the CLL samples. The controls and ZAP-70− samples appear together in group 4, but there was a clear separation between them (SI Appendix, Fig. S5).

The correlation among the levels of circulating miRNAs across different samples was further assessed by calculating the Pearson-moment correlation coefficient, r, using an internal tool, called “CoExpress.” As shown in SI Appendix, Table S4, four miRNA clusters were identified with r > 0.600 and P < 0.0001. A very strong correlation was observed between the levels of miR-93 and miR-198 (r = 0.916, P = 3.32E-16), two closely residing miRNAs on human chromosome 9q. The same correlation was seen for miR-15a and miR-16−, whose genes are close together on human chromosome 13q. Another large cluster, cluster 2, grouped miR-483–5p, miR-29a, and miR-150+ (r > 0.900 and P < 0.10E-12), but these miRNAs are not colocated in the genome.

**ZAP-70 Expression Status Affects the miRNA Spectra in Both Cells and Plasma.** Among the 27 miRNAs selected, most of them showed higher levels in the plasma of the CLL patients, compared with healthy controls, but most showed no significant changes in the corresponding B cells (Table 2). Even though the changes for some of the miRNAs, such as miR-150+, miR-638 in plasma, and
miR-181a in cells, correlate well with disease progression in ZAP-70+ samples, the majority do not show significant correlation with disease progression. Several miRNAs, such as miR-185, miR-221, miR-223, and miR-93, showed opposite changes between plasma and the corresponding cells, especially in the ZAP-70+ samples (SI Appendix, Fig. S6). This observation of reciprocal changes of miRNA between intracellular (tissue or cells) and extracellular (plasma) levels is similar to our previous findings in a drug-induced liver injury model (16). The difference between the two ZAP-70 groups of miRNAs compared with normals can be greater than 39-fold (for example, a 5.3 cycle number difference between ZAP-70+ stage III–IV and normal plasma for miR-29a). In general, the ZAP-70+ samples showed greater changes in the miRNA levels in both cells and plasma.

We also validated the changes of the most abundant miRNA species between ZAP-70+ and ZAP-70− pooled samples, miR-223 in ZAP-70+ and miR-150 in ZAP-70−, on individual plasma samples (31 patient and 17 healthy control samples) (SI Appendix, Fig. S7). Although the level of miR-233 was similar among all of the samples, there was a significant increase in the levels of miR-150 in ZAP-70+ plasma samples. The level of miR-150 increased with the severity of the diseases in ZAP-70+ samples, so there is staging information associated with this marker.

Assessing the Predictive Value of Circulating miRNAs in CLL Disease Stratification. The predictive power of changes in certain circulating miRNA levels was analyzed by receiver operating characteristic (ROC) curves and the associated area under the curve (AUC). The changes of circulating miR-195 (AUC = 0.951) or miR-20a (AUC = 0.910) levels were the best classifiers to separate CLL patients from healthy controls (Table 1). We tested the possibility of improving performance by combining the changes of several miRNAs. When all 14 miRNAs listed in Table 1 were combined, the AUC value derived from a standard principal component analysis (PCA) and ROC analyses reached 0.950. Excellent separation between CLL patients and controls can actually be reached by using only three of several strongly affected miRNAs—miR-195, -29a, and -222—in CLL patients; the AUC value reached 0.982 (SI Appendix, Fig. S8A).

The changes of miRNAs in plasma can also be used to predict the clinically important ZAP-70 expression status in CLL patients (Fig. 2). When combining the levels of several miRNAs—miR-29a, -483−5p, -195, -185, -135a, and -15a—it provided good separation between ZAP-70+ and ZAP-70− samples (AUC = 0.877) (SI Appendix, Fig. S8B). Among the miRNAs, the single best predictor was miR-29a (AUC = 0.90) for ZAP-70+ expression status. We also tested the possibility of using miRNA to assess the disease severity, the time interval from diagnosis to treatment need (the requirement for earlier treatment indicates more severe disease). Using the Kaplan–Meier method, the levels of miR-20a in plasma were shown to correlate well with the disease severity (P = 0.0242) (SI Appendix, Fig. S8C), which is similar to the value obtained by using ZAP-70 directly (P = 0.0205) (SI Appendix, Fig. S8D). On the basis of this finding, the level of miR-20a in plasma or positive ZAP-70 expression status in CLL cells, will need more aggressive and earlier treatment.

Some CLL cases are associated with mutated IgVH (17). We therefore examined the IgVH mutation status of 24 CLL patients directly by sequencing and compared this information with miRNA levels in plasma, but found no significant correlations. The levels of specific miRNAs in plasma were also compared with the absolute lymphocyte count (ALC) in the blood. The miR-29a, miR-150, miR-150*, and miR-483−5p levels in plasma showed strong correlations with ALC (r > 0.500 and P value < 0.01) on the basis of Pearson’s correlation coefficients (SI Appendix, Table S5).

Discussion

The presence of altered miRNA profiles in plasma has been reported for several types of solid tumors (13–15). In association with other markers, the changes in the levels of specific circulating miRNAs in plasma offer the potential for high sensitivity and specificity in tumor detection and classification (13, 18). Because access to plasma biomarkers is generally more feasible and simpler. We investigated here the spectra of miRNAs in plasma of CLL patients and find that specific circulating miRNAs could also be used to detect and classify CLL cases. We can extract a great deal of information about the presence, status, and stratification of CLL from plasma. Because miRNAs are exported from cells under some circumstances (19) the presence of altered miRNA species in plasma correlates well with the stage of the disease (more B cells in more severe disease stage). Consistent with our findings in CLL plasma, miR-150 was previously reported to increase in ZAP-70− CLL cells (20). Bone marrow stromal cells also provide key influences and protection for CLL B cells (21, 22), which suggests they may play a role in producing circulating miRNAs in B CLL patients. miR-451, -135a*, and -486−5p are more abundant in plasma (compared with B cells), which suggests that a significant fraction of these miRNAs in circulation were released by other cell types, perhaps including bone marrow stromal cells. This hypothesis raises an intriguing possible role for circulating miRNAs in the CLL. They could actually be acting as a communication signal between CLL B cells and other cell types including bone marrow stromal cells. Further investigations are needed to test this hypothesis.
Several microRNAs may affect key networks determining the pathology and prognosis of CLL cells. Many potential gene targets of miRNAs with altered expression patterns in either CLL cell or plasma (Table 2 and SI Appendix, Table S6) interact with proteins that are overexpressed in CLL cells such as Bcl-2, Mcl-1, p27, and Tcl-1 identified earlier (8, 23–25). miR-483-5p, elevated almost sixfold (2.5 Ct) in plasma of CLL patients, is predicted to target ERK1/MAPK3, which mediates IL-15-induced CLL proliferation (26) and drives CLL cell migration and infiltration (27). Although most of the miRNA–mRNA interactions are yet to be fully validated, the roles of these plasma miRNAs in CLL present intriguing biological questions with medically significant implications.

The spectrum of gene expression in the cell is regulated by both transcription factors and miRNAs. Alterations of the levels of either of these key elements can lead to profound changes in the gene expression profile in the cell. Two interesting pathological characteristics associated with the CLL B cells are the antiapoptotic properties associated with up-regulation of the BCL2 gene and shorter survival time with the expression of ZAP-70 gene in CLL B cells (28). To explore the involvement of miRNAs in these two important features of CLL pathology, we collected miRNA interacting gene target information from TargetScan Human V5.1 (http://www.targetscan.org), transcription factor binding site information from MSigDB (http://www.broadinstitute.org/cancer/gsea/index.jsp), and University of California Santa Cruz genome database (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/database) to build a hypothetical miRNA transcription factor–mediated regulatory network that could explain aspects of BCL2 and ZAP-70 expression.

A small hypothetical regulatory network, containing seven miRNAs and 13 transcription factors, was constructed, which might be involved in regulating the expression of BCL2 and ZAP-70 genes (Fig. 3A). The seven miRNAs in the network all showed reduced levels in CLL B cells (~1.5-fold decrease), especially in the ZAP-70+ samples, compared with normal controls. Among the seven miRNAs, miR-181a and miR-15a are predicted to interact with BCL2 directly or indirectly through transcription factors that may recognize the promoter region of the BCL2 gene. The miR-23b and miR-181a are predicted to interact with BCL2 and ZAP-70 through the transcription factor NR6A1 (SI Appendix, Fig. S9). Note that these circuits driving the expression of BCL2 and ZAP-70 represent coherent effects, which should exhibit switch-like behavior.

The NR6A1 receptor is encoded by 10 exons spanning a 240-kb region in the genome, and it is striking to note that two of the key miR-181 family member miRNAs, miR-181b-2 and miR-181a-2, are located within the NR6A1 gene in the second intron with transcription orientation opposite with respect to NR6A1 (Fig. 3B). The level of miR-181a in plasma showed little change, either between the ZAP-70 expression statuses or among different disease stages. However, in the CLL cells, the expression of miR-181a showed significant correlation among different disease stages in both ZAP-70+ and ZAP-70− samples (Table 2). Functional transcripts using the opposite strands of the same genomic sequence have been reported: for example, EIF2A and SERP1 on chromosome 3, CDSN and PSORS1C1 on chromosome 3, and TIMP3 and SYN3 on chromosome 22. The expression patterns of these miRNAs in the genome, and it is striking to note that two of the key miR-181 family member miRNAs, miR-181b-2 and miR-181a-2, are located within the NR6A1 gene in the second intron with transcription orientation opposite with respect to NR6A1 (Fig. 3B). The level of miR-181a in plasma showed little change, either between the ZAP-70 expression statuses or among different disease stages. However, in the CLL cells, the expression of miR-181a showed significant correlation among different disease stages in both ZAP-70+ and ZAP-70− samples (Table 2). Functional transcripts using the opposite strands of the same genomic sequence have been reported: for example, EIF2A and SERP1 on chromosome 3, CDSN and PSORS1C1 on chromosome 3, and TIMP3 and SYN3 on chromosome 22. The expression patterns of these”gene pairs” usually show negative correlations, probably because of regulatory effects and the possibility of forming unstable double-stranded RNA from the primary transcripts (29, 30). Lower miR-181a levels should then induce higher NR6A1 mRNA and protein levels and higher transcription rate of the NR6A1 gene. We examined this prediction, and a PCR measurement in ZAP-70+ and ZAP-70− samples confirmed the higher levels of NR6A1 transcription factor transcript in ZAP-70+ samples (Fig. 3C). Confirmation of this prediction is consistent with the proposed network.

About 40% of the miRNAs in the genome are located in the intronic regions of protein-coding transcripts (miRBase www.mirbase.org). Intronic miRNAs, like all miRNAs, play important roles in regulating various cellular functions. One of the most characterized intronic miRNA clusters is the miR-17–92 cluster, where miRNAs in this cluster played critical roles in normal tissue development as well as the oncogenesis of several types of cancers (31, 32). A recent report also showed that an intronic miRNA, miR-211, which is located in the intron 6 of melastatin, can promote the invasiveness of melanoma cells (33). Melastatin, a transient receptor potential calcium-channel family member, is highly expressed in melanomas in situ and down-regulated in metastatic melanomas. Silencing the melastatin and its intronic miRNA, miR-211, seems critical to suppress the metastatic potential for melanoma.

Most of the intronic miRNAs in the genome use the same strand of the genomic DNA and are cotranscribed with the host gene. Besides the example in NR6A1–miR-181 illustrated in this report, there are a few other examples, such as IARS2–miR-194 and DNM3–miR-199 that reside on different strands of the genomic DNA with respect to the host gene. It would be of great interest to

![Fig. 3. Putative regulatory network associated with BCL2 and ZAP-70 expression. (A) The genes and miRNAs involved in the putative regulatory network are indicated. The dashed lines indicate the miRNA–mRNA interaction and solid lines indicate the transcription factor–promoter interaction. The red color indicates overexpression of genes or miRNAs compared with normal; green color indicates lower expression. The miRNA expression levels in different ZAP-70 expression statuses are depicted in normal red circles; high indicates ZAP-70 expression status of the samples.](http://www.broadinstitute.org/cancer/gsea/index.jsp)
examine the expression correlation between these miRNAs and their host mRNAs. The finding of possible functional relationships between NR6A1 and miR-181 and melastatin and miR-211 suggests that these miRNAs play a regulatory role in the expression and processing of the intronic miRNAs and their host mRNAs. Because the levels of miR-181a went down in both ZAP-70+ and ZAP-70− samples (Table 2), it suggests the involvement of additional factors on the induction of NR6A1 and ZAP-70 genes. This preliminary evidence is therefore consistent with the model shown in Fig. 3 and suggests further hypotheses concerning the network biology of CLL.

The development and validation of miRNA biomarkers should have significant impact in improving early cancer detection, stratification of disease, enhancing therapeutic successes, and increasing the life expectancy of patients. For example, most tumors of a particular tissue can be stratified into distinct types—depending on the disease-perturbed combinations of networks they exhibit. This stratification into different tumor types will be important ultimately for achieving an impendence match with the proper therapy. We demonstrated here from a cohort of CLL patients the potential of using circulating miRNA for both detection of CLL and stratification of CLL patients. Levels for several miRNAs are strongly linked to cellular ZAP-70 expression status (Table 2). The time to treatment is an important clinical parameter (34) that also did have clear correlation with miRNA, miR-20a. These findings raise interesting questions about the origin and roles of circulating miRNAs in hematologic malignancies and suggest adopting circulating miRNAs as valuable biomarkers for CLL. Whether bone marrow stromal cells or CLL B cells constitutively produce and export miRNAs remains unclear and underlines the need to understand the biological origin and function of these circulating miRNAs. The resultant biology, including the network hypothesis put forward here, may clarify the therapeutic as well as diagnostic opportunities in the control of this disease.

Materials and Methods

Patients. Healthy controls and patients affected by hematologic malignancies were recruited from a single institution (Centre Hospitalier de Luxembourg) after obtaining a written informed consent in accordance with the Declaration of Helsinki. The number of patients who participated in the study are detailed in Dataset S1 and the clinical information of the CLL patients are described in SI Appendix, Table S1.

Plasma Preparation and B-Cell Isolation. All plasma samples were prepared by sequential centrifugations, detailed in Dataset S1, from whole blood collected in EDTA.

MicroRNAs Expression Profiling in RNA Pools. For miRNA profiling, total RNA was extracted from 300 μL of plasma with the miNeasy kit (Qiagen) as described before. The quality and quantity of RNA were assessed by using an Agilent 2100 Bioanalyzer. The RNA samples were pooled according to their disease stage by mixing equal amounts of RNA from each individual in the group. The miRNA profiles were conducted with Tag miRNA low-density array from Applied Biosystems. The detailed protocols for miRNA profiling and data analysis are described in Dataset S1.

Analysis of ZAP-70 and Sequence Analysis of IGHV. The ZAP-70 status was verified by RT-PCR as described before. The analysis of expressed IGHV gene was done by PCR followed by sequencing (98% homology as a threshold) as reported before.

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