microRNAome Expression in Chronic Lymphocytic Leukemia: Comparison with Normal B-cell Subsets and Correlations with Prognostic and Clinical Parameters

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\textbf{Abstract}

\textbf{Purpose:} Despite its indolent nature, chronic lymphocytic leukemia (CLL) remains an incurable disease. To establish the potential pathogenic role of miRNAs, the identification of deregulated miRNAs in CLL is crucial.

\textbf{Experimental Design:} We analyzed the expression of 723 mature miRNAs in 217 early-stage CLL cases and in various different normal B-cell subpopulations from tonsils and peripheral blood.

\textbf{Results:} Our analyses indicated that CLL cells exhibited a miRNA expression pattern that was most similar to the subsets of antigen-experienced and marginal zone–like B cells. These normal subpopulations were used as reference to identify differentially expressed miRNAs in comparison with CLL. Differences related to the expression of 25 miRNAs were found to be independent from IGHV mutation status or cytogenetic aberrations. These differences, confirmed in an independent validation set, led to a novel comprehensive description of miRNAs potentially involved in CLL. We also identified miRNAs whose expression was distinctive of cases with mutated versus unmutated IGHV genes or cases with 13q, 11q, and 17p deletions and trisomy 12. Finally, analysis of clinical data in relation to miRNA expression revealed that miR26a, miR532-3p, miR146-5p, and miR29c* were strongly associated with progression-free survival.

\textbf{Conclusion:} This study provides novel information on miRNAs expressed by CLL and normal B-cell subtypes, with implication on the cell of origin of CLL. In addition, our findings indicate a number of deregulated miRNAs in CLL, which may play a pathogenic role and promote disease progression. Collectively, this information can be used for developing miRNA-based therapeutic strategies in CLL. Clin Cancer Res; 20(15); 4141–53. ©2014 AACR.

\textbf{Introduction}

Chronic lymphocytic leukemia (CLL) is a disease of mature B cells and represents the most common leukemia in Western countries (1). Despite the rather homogeneous immunophenotype (CD19\textsuperscript{+}, CD20\textsuperscript{+}, CD5\textsuperscript{+}, and CD23\textsuperscript{+}), CLL is clinically heterogeneous. Some patients progress very slowly toward more advanced stages and may never require therapy or only after years from diagnosis whereas others require an early treatment. Despite the recent therapeutic advances, progressive CLL cases almost always become incurable diseases. The clinical outcome of CLL can be predicted on the basis of cellular and molecular markers, including the presence or absence of somatic mutations at the IGHV locus or ZAP-70 and CD38 expression in neoplastic cells (1). Cyto genetic aberrations, including 17p and 11q deletions as well as trisomy 12, have also been related to poor prognosis (2). More recently, mutations in TP53, NOTCH1, SF3B1, and BIRC3 genes were shown to predict poor outcome (3).
Translational Relevance
Evidence indicated a role for miR15/16 in chronic lymphocytic leukemia (CLL) pathogenesis, suggesting the involvement of additional deregulated miRNAs. However, the pattern of deregulated miRNAs in CLL could be related to a number of factors, including presence/absence of chromosomal abnormalities, disease stage, and, importantly, types of comparator normal B cells. Here, we performed miRNA profiling in a prospective cohort of 217 early-stage CLL in comparison with B cells from different normal subsets. Our data demonstrated that CLL have a miRNA expression profile most similar to that of antigen-experienced B cells, allowing the identification of a 25-miRNA signature specifically associated with the disease. Some of these miRNAs were likely to play a role in disease progression given their observed correlation with clinical course. Finally, these miRNAs were different from those found to be deregulated in CLLs with known chromosomal abnormalities. Such an approach may help in the design of miRNA-based therapeutic strategies in CLL.

Materials and Methods
Patients, CLL cell preparation, and prognostic marker determination
Patients with CLL from several institutions were enrolled in the O-CLL1 protocol (clinicaltrial.gov identifier NCT00917540). Exclusion criteria were: (i) CLL diagnosis more than 12 months before registration; (ii) leukemic phase lymphoproliferative disorders of B cells with a CD5+ and/or CD23+ cell surface phenotype according to flow cytometric analysis; (iii) clinical Binet stage B or C; (iv) need of therapy according to NCI guidelines; and (v) age ≥ 70 years. Diagnosis was confirmed by flow cytometric analysis, together with the determination of CD38 and ZAP-70 expression and IGHV mutational status. Peripheral blood mononuclear cells from patients with CLL were isolated by Ficoll-Hypaque (Seromed, Biochrom KG) density gradient centrifugation. For miRNA expression profiling, CLL cells were enriched by negative selection with the EasySep-Human B Cell Enrichment Kit without CD43 depletion (Stem Cell Technologies, Voden Medical Instruments) using the fully automated protocol of immunomagnetic cell separation with RoboSep (Stem Cell Technologies). The proportion of CD5/CD19/CD23 triple positive B cells was determined by direct immunofluorescence with mAbs to CD19-FITC (BD Biosciences Pharmigen), CD23-PE (BD Biosciences, BD), and CD5-PC5 (Beckman Coulter Immunotech). ZAP-70 expression was determined by flow cytometry with a ZAP-70 FITC (clone 2F3.2, Millipore) or an isotype control mAb (mouse IgG2a FITC BD Biosciences) as previously described (11). To assess IGHV gene mutational status, DNA sequences from pathological samples were aligned to IMGT and analyzed using IMGT/VQUEST software. Sequences differing more than 2% from the corresponding germline gene were considered mutated. Deletions at chromosomes 11q23, 13q14, and 17p13 and trisomy 12 were evaluated by FISH in purified CD19+ population as previously described (12).

Normal B-cell populations
Normal B lymphocytes were obtained from peripheral blood buffy coats or tonsils. Tonsil samples were first finely minced in normal culture medium of RPMI-1640 containing 10% FBS (Invitrogen) and passed through a cell strainer (BD) with a 70-μm grid to obtain single-cell suspensions. B cells from buffy coats or tonsils were first enriched by removing cells forming rosettes with sheep red cells (T cells) using RosetteSep Human B-cell enrichment cocktail (Stem Cell Technology). The remaining cells were then sorted by FACS (FACS Aria, Becton Dickinson) using allophycocyanin (APC)-labeled anti-CD19 mAb (BD). To study different B-cell subsets, purified B cells from buffy coat and tonsils were stained with the following combination of antibodies: FITC-labeled polyclonal anti-δ (Dako), Pe-Cy5...
or PE-CF594-labeled anti-CD27 mAb (BD), or PC7-labeled anti-CD38 mAb (BD). CD19+ B cells from buffy coats, depleted of CD38 to exclude plasma cells (called B cells or BC), were further sorted into IgDbrightCD27− B cells (naïve B cells), IgDlowCD27+ cells (IgM-memory or IgM-mem B cells), and IgD− CD27+ cells (switched memory or SM B cells). Because of the paucity of B cells, the 2 samples included in the analyses represent pools of cell subset populations from 2 and 3 buffy coats, respectively. Tonsil CD19+ B cells were separated further based on the expression of IgD versus CD38 molecules. The following nonadjacent gates were drawn to separate naïve B cells (IgDbrightIgMbrightCD38−CD27−), marginal zone–like B cells (IgDlowIgMbrightCD38−CD27+), germinal center (GC) B cells (IgD−CD38+CD24−), and switched memory (SM) B cells (CD19+IgD−CD38+). The gates used for FACS sorting are reported in Supplementary Fig. S1.

Agilent miRNA microarrays and data analysis
Total RNA fraction was obtained using the TRIzol reagent (Life Technologies, Invitrogen). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Low-quality RNAs (integrity < 7) were excluded from microarray analyses. RNAs were hybridized on an Agilent Human miRNA microarray (G4470B, Agilent Technologies), which consists of 60-mer DNA probes synthesized in situ and contains 15,000 features specific for 723 human miRNAs (Sanger miRBase public database, Release 10.1). One-color miRNA expression was performed according to the manufacturer’s procedure. Microarray results were analyzed using the GeneSpring GX software (Agilent Technologies) or the Qlucore Omics Explorer (Qlucore). Quantile normalization was used. A filter on low miRNA expression was used so that only the probes expressed (flagged as present) in at least one sample were kept; samples were grouped in accordance to their various conditions and comparative analyses performed. In general, differentially expressed miRNA were selected as having a 2-fold expression difference between their geometrical mean and a statistically P value < 0.05 by an unpaired t-test statistic, followed by the application of the Benjamini–Hochberg correction for false-positives reduction. Differentially expressed miRNA were used for cluster analysis of samples, using the Pearson correlation as a measure of similarity. The multivariate principal component analysis (PCA) was used to reveal internal structure of the data in 3-dimensional space. In addition to this traditional approach, Qlucore Omics Explorer software was used to visualize the best clustering of the different classes and reveal the set of associated miRNAs. Also in this case, the Benjamini–Hochberg correction for false-positives reduction was applied. Microarray data were submitted to the Array Express public database (accession number: E-MTAB-1454).

Statistical analyses
For comparing the expression of individual miRNAs in various groups, P values were calculated using an unpaired t test with Welch correction using the GraphPad Prism software (GraphPad Software Inc.). The correlation coefficients between classes were calculated using the Pearson correlation coefficient (GeneSpring GX software, Agilent Technologies and GraphPad Software). For analysis of progression-free survival (PFS), the statistical package SPSS Statistics 19 (SPSS Inc. and IBM, 2010) was used. Cases were grouped by median values according to miRNA expression and their prognostic impact on PFS, defined as the time to therapy need, was investigated by univariate Cox regression analysis. The independent relationship between each miRNA that resulted significant in univariate model and the outcome variable was investigated by Cox multivariate models adjusting for the following potential confounders: CD38 and ZAP-70 expression, FISH data, and IGHV mutational status. Data are expressed as HRs and 95% confidence interval (CI). P ≤ 0.01 was considered significant for statistical calculations. Benjamini and Hochberg method was used for multiple test corrections.

Results

miRNA expression profiles in CLL patients
Two hundred and seventeen patients with Binet stage A CLL were investigated. Their specific features are summarized in Supplementary Table S1. Most of the cases were Rai stage 0. Patients were classified as mutated (M-CLL) or unmutated (UM-CLL) based on the presence of IGHV somatic mutations. They were also characterized for ZAP-70 and CD38 expression and the presence of major chromosomal aberrations (deletions at 13q, 11q, and 17p as well as trisomy 12).

Merely to ascertain the homogeneity of miRNA expression in CLL and exclude possible outliers, sets of unselected CLL, multiple myeloma, and Sezary syndrome samples were investigated. A cluster analysis was performed using expressed miRNAs (average log expression > 0 in at least one class), selected as differentially expressed in a multiclass ANOVA analysis (P < 0.01). The results of the analysis, shown as heatmap and PCA in Supplementary Fig. S2, indicate that CLL displays a relatively homogeneous pattern of miRNA expression, clearly distinct from other lymphoproliferative disorders. Four CLL samples were ascertained to be outliers and excluded from all subsequent analyses, which were therefore performed on 213 samples.

Comparison of miRNA expression profiles of CLL cell with normal B-cell subsets
We first established those B-cell subsets that shared miRNA expression similarities with CLL cells. To address this issue, various subpopulations of normal B cells from peripheral blood and tonsils were isolated as described in Materials and Methods. They included peripheral blood CD19+ CD38− B cells (B cells depleted of plasma cells) as well as naïve, IgM-memory, and switched memory B cells. The following B-cell subpopulations from tonsils were used: germinal center, naïve, marginal zone–like, and switched memory B cells. Supplementary Figure S1 describes the FACS sorting strategy used to purify these cell subpopulations. Peripheral blood B cells included 7 samples of buffy coat, 3 samples of naïve B cells, and 2 samples each of IgM-
memory and switched memory B cells, with each sample representing a pool derived from 2 or 3 buffy coats. Tonsil samples included 4 germinal center, 5 naïve, 4 marginal zone–like cells, and 5 switched memory B cells, each sample being a pool of cells obtained from 2 or 3 tonsils. On the basis of PCA and unsupervised cluster analysis, a set of 50 miRNAs produced the best discrimination between the different subsets of normal B cells (Fig. 1A and B). The patterns of miRNA expression of IgM-memory and switched memory B cells from peripheral blood and of marginal zone–like and switched memory B cells from tonsils were almost identical. Peripheral blood and tonsil naïve B cells also exhibited a miRNA profile very similar to these B cells, whereas tonsil germinal center B cells and peripheral blood buffy coat exhibited a fairly divergent pattern. The same set of 50 miRNAs was then used to compare the miRNA expression of normal B-cell subsets to that of CLL cell samples. PCA analysis revealed that CLL were more similar to tonsil marginal zone–like (5 samples) and switched memory B cells (5 samples) with that of normal B cells including tonsil marginal zone–like (5 samples) and switched memory B cells (5 samples). Given the similarities of miRNA profile of these normal subpopulations (collectively, antigen-experienced B cells plus marginal zone–like B-cells), these could be combined for comparisons with CLL cells and will be denominated henceforth as “comparator” cells. When the miRNA expression profiles of the cells from an unselected cohort of 99 CLL cases (training set) was compared with the comparator cells, 106 miRNAs differentially expressed between the 2 groups were identified (P < 0.01; Supplementary Table S2). Unsupervised cluster analysis produced an excellent discrimination between CLL and normal B cells (not shown). Of the 106 miRNAs differentially expressed by CLL cells and normal comparator cells, 25 remained distinct in any comparison carried out with individual FISH subgroup of CLLs (Table 1 and data not shown).

**Figure 1.** Comparisons of miRNA expression pattern in CLL cells and various normal B-cells subsets. A, heatmap of an unsupervised cluster analysis based on a 50-miRNA expression profile that best discriminate the normal B-cell subsets. B, similar to the cluster analysis, a PCA shows that antigen-experienced B cells, inclusive of peripheral blood IgM-memory and switched memory (SM) B cells and of tonsil SM B cell, marginal zone (MZ) cells, and naïve B cells are closed in a 3-dimensional space of similarity, whereas buffy coat (BC) and germinal center (GC) are more distant. C, a PCA analysis of CLL samples shows that CLLs are closer to antigen-experienced B cells + MZ-like B cells and naïve B cells than to other B-cell types. D, correlation plot showing coefficients for each pair of arrays displayed in a textual form as a table as well as in a heatmap form. The correlation coefficient was calculated by the Genespring software using the Pearson correlation coefficient. BC buffy, peripheral blood CD19+ B lymphocytes; MEM, memory B cells; naïve, naïve B cells; SE, subepithelial B cells; Tons, tonsil.
Table 1. MicroRNAs commonly dysregulated in CLL versus normal "comparator" B cells

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<th>Fold changec</th>
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<th>CLL expression</th>
<th>Memory B-cell expression</th>
<th>active_sequence</th>
<th>chr</th>
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<th>Stop</th>
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aSelected test: t test unpaired; P value computation: asymptotic.
bMultiple testing correction: Benjamini–Hochberg; Corrected P cutoff = 0.05.
cFold change cutoff: 1.5.
Because of the concordant deregulation in any CLL subgroup, this shorter list may include miRNAs involved in the initial steps, rather than progression, of CLL pathogenesis. The 25 miRNAs signature maintained an excellent discriminating power between CLL and normal (antigen-experienced $+$ marginal zone–like) B cells (Fig. 2); notably, it maintained an excellent discriminating capacity when applied to a second independent CLL group (validation set, $n = 114$; Fig. 2). Clinical and biomolecular characteristics between CLL training and validation sets did not differ significantly (Supplementary Table S1).

Some of the discriminating miRNAs, miR125a-5p, miR130a, miR365, miR193b, and miR26a, were further validated on a third group of independent unselected CLLs ($n = 20$) by using a quantitative real-time PCR (qRT-PCR). All the analyses confirmed to a great extent the microarray results (Supplementary Fig. S3); only miR181b, which showed a significant downregulation in microarray studies, did not achieve a significant difference in PCR analyses, likely because of the wide distribution of expression levels among samples.

Figure 2. miRNA expression in CLLs versus normal B cells. Unsupervised cluster and PCA analyses based on the miRNAs are shown in Table 1. Top, the analyses based on the CLL training set of samples; bottom, the analyses of the independent validation set. Both sets exhibit an excellent separation between CLL (blue label in top; cyan label in bottom) and "comparator" B cells (peripheral blood (PB) switched memory (SM) B cells $+$ PB IgM-memory B cells $+$ tonsil SM B cells $+$ tonsil marginal zone (MZ)-like B cells; green label in both panels).

miRNA expression in CLL subgroups stratified according to IGHV gene somatic mutations

We next compared miRNA expression of M-CLL ($n = 131$) versus UM-CLLs ($n = 82$). The analysis identified 14 differentially expressed miRNAs based on selection criteria of an average log$_2$ expression $> 0.0$ in at least one of the classes, fold change $> 1.5$, and the Welch $t$ test $P < 0.05$ (Table 2). Because the results based on all CLL samples could have been influenced by heterogeneity of samples (i.e., the different distribution of cytogenetic classes in the 2 IGHV classes), we also performed the same M-CLL versus UM-CLL analysis on the more homogeneous CLL subgroup with deletion 13q as the sole abnormality, which included 70 M-CLLs and 15 UM-CLLs. A list of 7 differentially expressed miRNAs was obtained (Table 2), which were also found in the initial analysis based on all CLL samples.

Among the miRNAs differentially expressed in UM-CLL versus M-CLL samples, miR29c* and miR146b-5p possessed the most significant $P$ values. The low miR29c* expression was the feature most closely related to the UM-IGHV phenotype (Supplementary Fig. S4A) and its...
Table 2. Differentially expressed microRNAs between M-CLL and UM-CLL

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<th>13q(^b)</th>
<th>Corrected(^c)</th>
<th>Fold change(^d)</th>
<th>Regulation</th>
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<th>CLL [UM] expression</th>
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\(^a\)133 M-CLLs versus 82 UM-CLLs.
\(^b\)70 M-CLLs versus 15 UM-CLLs.
\(^c\)Selected test: \(t\) test unpaired; \(P\) value computation: asymptotic.
\(^d\)Multiple testing correction: Benjamini–Hochberg; Corrected \(P\) cutoff = 0.05.
\(^e\)Fold change cutoff: 1.5.
expression per se provided a tool to distinguish between M-CLL and UM-CLL (Supplementary Fig. S4B). Notably, the miR29c/29c* expression levels in CLL versus the "comparator" cells revealed an unexpected scenario: both miRNAs exhibited an average expression above normal in the M-CLL group and below normal in the UM-CLLs (Supplementary Fig. S4C). This finding may explain why miR29c/29c* did not reveal a significant difference between all CLL cases and "comparator" cells.

Some of the other miRNAs discriminating UM-CLL versus M-CLL, namely miR146b-5p, miR29c, miR625, and miR532-3p, were further analyzed with qRT-PCR providing good and significant correlation coefficients with microarray analysis (Supplementary Fig. S5).

**miRNA expression by CLL cells with defined cytogenetic aberrations**

To identify characteristic patterns of miRNA expression associated with each of the major cytogenetic aberrations, we compared miRNA expression in the different cytogenetic CLL subgroups. FISH “negative” CLLs were considered as a single subgroup; when 13q/-13q was present together with another cytogenetic abnormality, we considered the latter as predominant in determining group inclusion. By selecting the top miRNAs differentially expressed in each cytogenetic subgroup versus all other CLLs and applying a Kruskal–Wallis test to confirm that they were the best-performing miRNAs, we generated a short list of miRNAs that could be specifically associated with each FISH group (Table 3). The cluster analyses shown in Supplementary Fig. S6 visually represent the average pattern of expression of these miRNAs in each class versus the other CLL classes (right) or versus normal “comparator” cells (left). The clearest results included the downregulation of miR34a and the upregulation of miR96 and miR21* in 17p− CLls; the upregulation of miR338-3p in the 11q− class; the down-regulation of miR148a, miR21*, miR155, and miR483-5p in trisomy 12 cases; and the downregulation of miR16 in 13q− cases, more evident in patients with biallelic deletion.

Validation based on qRT-PCR was performed only for miR146b-5p (see Supplementary Fig. S5) because the downregulation of miR34a in the 17p− CLL cases (13, 14) and the downregulation of miR16 in the 13q− cases had already been confirmed by earlier reports (15).

**Assessment of the predictive clinical value of deregulated miRNAs**

Next, we assessed the prognostic impact on PFS of miRNA lists reported in Tables 1 to 3. Complete clinical information was available for 193 patients with a median follow-up time for all patients of 42 months (range, 1–80 months). Fifty-eight CLL cases progressed and required therapy. Within the list of miRNAs in Table 1, the low expression of 5 miRNAs (miR26a, miR532-3p, miR532-5p, miR502-3p, and miR660) was significantly associated with a shorter PFS. Among the 14 miRNAs in Table 2, nine showed significant association with PFS (Fig. 3). In particular, CLL cases with miR29c*, miR532-3p, miR146b-5p, miR139-5p, miR222, and miR29c exceeding the median values showed a reduced risk of disease progression, whereas higher expression of miR2338-3p, miR574-3p, miR16 exhibited an unfavorable clinical outcome. Finally, among the 17 miRNAs in Table 3, 4 were significantly associated with PFS (Fig. 3): high expression of miR146b-5p appeared to have a protective effect from disease progression, whereas high expression of miR155, miR338-3p, and miR16 had an opposite effect. Overall, 11 of the 18 miRNAs associated with PFS retained significance after multiple test adjustments (Fig. 3); after data adjustment for confounders CD38 and ZAP-70 expression, FISH data, and IGHV mutational status, only 2 miRNAs, miR26a (HR, 0.41; 95% CI, 0.23–0.73; \( \hat{P} = 0.002 \)) and miR532-3p (HR, 0.46; 95% CI, 0.25–0.82; \( \hat{P} = 0.008 \)), remained significantly associated with PFS.

**Discussion**

In this study, cells from a large cohort of patients with Binet A stage CLL were investigated for miRNA expression profiling and the data compared with those obtained for various normal B-cell subpopulations. The observations strongly support the conclusion that CLL miRNA expression signature is most similar to that of normal antigen-experienced cells, which included IgM-memory and switched memory B cells from peripheral blood and switched memory B cells from tonsils. In addition, tonsil marginal zone–like B cells exhibited an miRNA signature very similar to that of tonsil switched memory B cells and of CLL cells, a finding not unexpected considering that marginal zone–like B cells are mostly antigen-experienced B cells and home in the same anatomical area as switched memory B cells (16). Other B-cell subsets, like circulating buffy coat and of germinal center B cells from tonsils, exhibited a divergent miRNA signature. Naïve B cells from both peripheral blood and tonsils displayed close similarities with antigen-experienced B cells, although the differences with the CLL cell signature were more pronounced. Although with somewhat different procedures, Basso and colleagues reached the same conclusions regarding similarities of miRNA signatures among the various human B-cell subsets (17).

The notion that CLL cells are antigen-experienced B cells is also supported by additional evidence: (i) the presence of somatic IGHV hypermutation in M-CLL cases and (ii) the utilization of a stereotyped IGHV/IGHL gene repertoire by CLL compared with normal B cells. These considerations are in agreement with a definition of antigen-experienced cell for the CLL cell and with our miRNA data. It is also of note that Klein and colleagues found that memory B cells were most similar to CLL cells using gene expression profiling technologies (18). However, evidence for a heterogeneous origin of CLL cells from CD5− B-cell subpopulations (CD27− or CD27+) was recently presented (19). Yet, previous studies indicated that normal CD5− B cells exhibit rather unique miRNA and gene expression profiles compared with CLL (6, 18).

The above data provide clues regarding the nature of the CLL cell of origin. However, a note of caution should be...
Table 3. MicroRNAs deregulated in specific FISH subgroups of CLL

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<th>Average expression in FISH class</th>
<th>Average expression in all other CLLs</th>
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<th>Regulation</th>
<th>Corrected (P^{ab})</th>
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*aSelected test: t test unpaired; P value computation: asymptotic.

*bMultiple testing correction: Benjamini–Hochberg; Corrected P cutoff = 0.05.
added: the similarities with antigen-experienced B cells may only reflect the maturation stage reached by CLL cells in the context of a leukemic stem cell hierarchical model (20). On the other hand, the cancer stem cell compartment of mature B-cell malignancies might reside within memory B-cells, which have self-renewing potential (21), suggesting that the present data might lead to the identification of the maturation stage at which the latest transformation event(s) took place (22).

The comparison between CLL cells and the most similar normal "comparator" cells led to the identification of 106 deregulated miRNAs, some of which emerged as deregulated in previous studies that used unsorted circulating B cells as comparators (7, 9, 10); in particular, upregulation of miR155 and miR150 (7, 9, 10) was observed as well as the downregulation of miR181b (10), miR222, and miR92 (7). Our results established that miR34a is generally upregulated in CLL, as reported by Li and colleagues (10), but also confirmed that it is strongly downregulated in CLL cases with deletion at 17p (14, 23). However, other miRNAs were found to be deregulated using the specific approach reported herein, including miR193b, miR33b, and miR196 among the downregulated miRNAs and miR23b, miR26a, and miR532 (5p and 3p) among the upregulated ones. It should be noted, however, that most of the previous studies on miRNA expression were either performed in particular subsets of CLL samples (24–28) or were focused on specific miRNAs (29–32). Hence, comparison with our study is difficult although similar results were obtained in certain instances, such as the downregulation of miR34a and the upregulation of miR155 (25, 29) and miR21 in 17p– cases (28). The downregulation of miR181b in CLL versus normal B cells was confirmed, although no association with disease progression was found. Conversely, our findings did not confirm the deregulation of miR650 or miR17-92 family in CLL.

On the basis of our analyses, the number of deregulated miRNAs in CLL appears to be very large, which suggests that it is unlikely that all are involved in the pathogenesis of the disease. One can speculate that the deregulation of several miRNAs could be related to Dicer dysfunction, as previously reported (33), or related to the fact that CLL cells are constitutively activated in vivo as demonstrated by the expression of surface activation markers. However, some of them may still be relevant for CLL pathogenesis, although functional studies are required to formally demonstrate a specific role in leukemogenesis. In particular, previous reports indicated that the downregulation of miR181 and miR193b and the upregulation of miR26a, miR125a-5p, miR130a, and miR155 were traits common to various malignancies, including CLL (10, 29, 34–38). The functional significance of their aberrant expression could be linked to the disrupted expression of some important cancer-associated gene targets, including Bcl-2, MCL1, KRAS, and TCL1 for miR181b (35, 39, 40); c-KIT, MCL1, cyclin D1, and ETS1 for miR193b (41). The miR130a was also recently shown to control a survival pathway in CLL by targeting ATG2B and DICER1 and inhibiting autophagy (32). In support of their pathogenic significance, it is of interest that the deregulation of certain miRNAs, like miR29c, miR29c, miR26a, miR532-3p, miR146b-5p, miR139-3p, miR222, miR338-3p, miR574-3p, miR155, and miR16, was associated with a shortened PFS in our series, possibly indicating a role of these miRNAs in disease progression.

It should be noted that some of the above findings may appear counterintuitive. For example, the upregulation of miR34a supports the concept that apoptosis is active in the majority of CLLs, and maintenance of the leukemic clone requires a significant level of cell replication. These findings are in agreement with previous studies indicating that CLL cells have high apoptosis and cell proliferation.
levels (42). When this equilibrium is disrupted, as in CLL cases carrying a 17p deletion, leukemia becomes more aggressive and patient’s prognosis poor. A similar scenario may also be claimed for miR130a recently shown to control a survival pathway in CLL by targeting ATG2B and DICER1 and inhibiting autophagy (32). While we confirmed (data not shown) that miR130a exhibits a reduced expression in CLL cells when CD19+ peripheral blood cells are used as comparators (32), miR130a levels were higher than normal with antigen-experienced B cells as comparators, a condition expected to inhibit autophagic survival and favor cell death in malignant cells. The existence of active cell death mechanisms may perhaps justify the indolent nature of CLL.

We also identified miRNAs whose altered expression was related to the presence of defined chromosomal aberrations. Specifically reduced miR16 expression was significantly associated with biallelic and monoallelic 13q deletion, in accordance with the notion of a gene dosage effect for the expression of this miRNA (15). The down-regulation of miR34a was closely related to the presence of 17p deletion (14, 23). Because miR34a expression is under the transcriptional control of p53 (see ref. 43 for a recent review), this result suggests that deletion at 17p, where the TP53 gene is located, may influence downstream TP53 targets, including miR34a (14, 23). Also of note, it is the observed downregulation of miR155 in CLLs with trisomy 12, in agreement with a previous report (9). However, this may represent an odd evidence, given that miR155 is one of the most well-established oncomirs in hematologic and solid tumors (44, 45) and is found upregulated in most CLLs, in particular in 17p− and 11q− cases.

M-CLL and UM-CLL cases displayed a very similar miRNA signature, in agreement with the data of earlier studies (19, 46), although with some exceptions. For example, miR29c+ and its partner miR29c could discriminate cases from the 2 groups. Both miRNAs were downregulated in the UM-CLL poor prognosis group. It has been reported that members of the miR29 family have the ability of downmodulating the expression of a number of oncogenes such as TCL-1, MCL-1, and CDK-6, which are possibly involved in CLL and mantle cell lymphoma; hence, this observation is of potential pathogenetic relevance (40, 47) and is in agreement with previous reports (4, 9, 10, 47, 48). It is also of note that miRNA-29c was found to be consistently upregulated in CLL cells by Lawrey and colleagues who did not, however, subdivide their cases into M- and U-CLL (9). Indeed, in our study, miR29 (mainly miR29c and miR29c+) exhibited a weak upregulation in M-CLLs compared with normal B cells. Overall, these observations suggest that miR29 oncogenic activity could depend upon cellular background (49): the upregulation may support clonal expansion in more indolent CLL cells, as it has also been described for miRNA-29b in a form of indolent mouse lymphoma (50) and promotes an aggressive form of CLL when downregulated.

Finally, some of the miRNAs associated with cytogenetic abnormalities or IGHV mutational status were also associated with PFS by univariate analyses. The high expression of miR29c, miR532-3p, miR146b-5p, miR139-3p, miR222, and miR29c was associated with a reduced risk of disease progression, whereas the opposite was true for miR338-3p, miR574-3p, and miR16. Likewise, among the miRNAs associated with cytogenetic abnormalities, high levels of miR146b-5p appeared to have a protective effect from disease progression, whereas the opposite was observed for miR155, miR338-3p, and miR16. Some of these miRNA (miR29c, miR146, miRNA-222, and miR155) were included in an earlier miRNA-based signature associated with PFS prediction (4). However, among all the above, only miR26a and miR532-3p (found to be significant in the comparison of CLL with normal memory B cells) maintained a predictive value after adjustment for covariate confounders, such as FISH analyses, IGHV mutational status, and ZAP-70 or CD38 expression in multivariate analyses.

In conclusion, the present study, which includes a large cohort of CLL together with a variety of normal cellular comparators and various validation approaches, provides a comprehensive picture of miRNA signatures in CLL. It also gives indications on the potential role of certain miRNAs in disease pathogenesis and progression. This information has relevance for strategies aimed at taking advantage of miRNAs in therapeutics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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