**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+ , CD20+, CD5+, and CD23+), 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). Cytogenetic aberrations, including 17p deletions and 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). In
found distinct associated patterns of differentially expressed according to their molecular and cytogenetic markers, we
CLL were stratified in different prognostic subgroups
potential pathogenic value. In addition, when patients with
found to be differentially expressed, which may have a
zone–like B cells displayed very similar signatures and were
vinal zone–like B cells. Antigen-experienced and marginal
enced B cells from tonsils and peripheral blood and mar-
mRNA profiles of CLL cells and those of antigen-experi-
duced rather heterogeneous results for various reasons,
addition, expression of certain miRNAs by leukemic cells can
provide useful information of prognostic relevance (4).
mRNAs are short, noncoding RNAs that play a key role in
posttranscriptional regulation of gene expression. Their
aberrant expression is common in malignancy and may be
relevance for disease onset and progression. Importantly,
recent studies have suggested the possibility of establishing
miRNA-based therapeutics, either by restoring tumor-sup-
pressive miRNAs or by inhibiting miRNAs with pathogenic
functions (5).
Abrerrantly expressed miRNAs in CLL have been reported
by various expression profile studies (6–10), which pro-
duced rather heterogeneous results for various reasons,
including the size and characteristics of the CLL series, the
different normal B-cell populations used for comparison,
and the use of different platforms for miRNA quantification.
In the present study, we used a commercial microarray
platform to investigate leukemic cells from a large cohort
of patients with Binet stage A CLL and compared their
profile with various subsets of normal B-cell populations
purified from tonsils or peripheral blood.
Our study demonstrated marked similarities between the
miRNA profiles of CLL cells and those of antigen-experi-
cenced B cells from tonsils and peripheral blood and mar-
ginal zone–like B cells. Antigen-experienced and marginal
zone–like B cells displayed very similar signatures and were
used for identifying differentially expressed miRNAs in
comparison with CLL cells. Indeed, several miRNAs were
found to be differentially expressed, which may have a
potential pathogenic value. In addition, when patients with
CLL were stratified in different prognostic subgroups
according to their molecular and cytogenetic markers, we
found distinct associated patterns of differentially expressed
miRNAs. These observations may provide new opportu-
nities for investigating miRNA-based prognostic stratifica-
tion and therapeutic approaches in CLL.

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 pres-
ence/absence of chromosomal abnormalities, disease
stage, and, importantly, types of comparator normal B
cells. Here, we performed miRNA profiling in a prospec-
tive cohort of 217 early-stage CLL in comparison with B
cells from different normal subsets. Our data demon-
strated 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 deregu-
lated 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) den-
sity gradient centrifugation. For miRNA expression profil-
ing, CLL cells were enriched by negative selection with the
EasySep-Human B Cell Enrichment Kit without CD43
depletion (Stem Cell Technologies, Voden Medical Instru-
mants) using the fully automated protocol of immunomag-
netic cell separation with RoboSep (Stem Cell Technolo-
gies). 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, Milli-
pore) or an isotope 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 either periph-
oral 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 sus-
pensions. B cells from buffy coats or tonsils were first enrich-
ed by removing cells forming rosettes with sheep red cells
(T cells) using RosetteSep Human B-cell enrichment cock-
tail (Stem Cell Technology). The remaining cells were then
sorted by FACS (FACS Aria, Becton Dickinson) using allo-
phytocyanin (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

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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 significant 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 ofuffy 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 B cells and to the group of antigen-experienced B cells, including peripheral blood IgM-memory and switched memory B cells and tonsil switched memory B cells. Naïve B cells from peripheral blood and tonsils also were more similar to CLL cells than tonsil germinal center B cells or peripheral blood buffy coat (Fig. 1C). In a second approach, a correlation analysis using the Pearson correlation coefficient, based on those miRNAs (n = 330), the expression of which was above background in at least 1 sample, revealed that the normal B-cell populations most similar to CLL were marginal zone–like B cells (correlation coefficient = 0.472) and switched memory+ naïve B cells (correlation coefficient = 0.415) B cells. Naïve B cells exhibited a moderate correlation (correlation coefficient = 0.395), whereas markedly lower values were obtained for peripheral blood buffy coat (correlation coefficient = 0.237) and tonsil germinal center B cells (correlation center = 0.231; Fig. 1D).

Differentially expressed miRNAs possibly involved in CLL pathogenesis

We next compared the CLL miRNA expression profile with that of normal B cells, including tonsil marginal zone–like (5 samples) and switched memory B cells (5 samples), peripheral blood IgM-memory (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

<table>
<thead>
<tr>
<th>miRNA</th>
<th>CLL</th>
<th>Naïve</th>
<th>GC</th>
<th>MEM</th>
<th>MZ</th>
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</thead>
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<tr>
<td>B</td>
<td>0.237</td>
<td>0.472</td>
<td>0.442</td>
<td>0.421</td>
<td>0.840</td>
</tr>
<tr>
<td>GC</td>
<td>0.232</td>
<td>0.472</td>
<td>0.442</td>
<td>0.421</td>
<td>0.840</td>
</tr>
<tr>
<td>MEM</td>
<td>0.177</td>
<td>0.442</td>
<td>0.421</td>
<td>0.421</td>
<td>0.840</td>
</tr>
<tr>
<td>MZ</td>
<td>0.041</td>
<td>0.472</td>
<td>0.442</td>
<td>0.421</td>
<td>0.840</td>
</tr>
<tr>
<td>Naïve</td>
<td>-0.505</td>
<td>0.395</td>
<td>0.670</td>
<td>0.772</td>
<td>1.000</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Corrected P(^{ac})</th>
<th>Fold change(^b)</th>
<th>Regulation in CLL</th>
<th>CLL expression</th>
<th>Memory B-cell expression</th>
<th>active_sequence</th>
<th>chr</th>
<th>Start</th>
<th>Stop</th>
<th>miRbase accession</th>
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<tbody>
<tr>
<td>hsa-miR193b</td>
<td>4.32E-55</td>
<td>6.49</td>
<td>Down</td>
<td>1.05</td>
<td>6.83</td>
<td>AGCGGGACCTTGAGGG</td>
<td>chr16</td>
<td>14,397,881</td>
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<td>14,397,895</td>
</tr>
<tr>
<td>hsa-miR365</td>
<td>2.51E-36</td>
<td>4.44</td>
<td>Down</td>
<td>0.81</td>
<td>3.60</td>
<td>ATAGGATTGTAGGGACGTA</td>
<td>chr16</td>
<td>14,403,197</td>
<td>..</td>
<td>14,403,218</td>
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<tr>
<td>hsa-miR33b(^c)</td>
<td>7.10E-31</td>
<td>5.92</td>
<td>Down</td>
<td>0.87</td>
<td>5.15</td>
<td>GGCGGCTGACTGCG</td>
<td>chr17</td>
<td>17,717,224</td>
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<td>17,717,212</td>
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<tr>
<td>hsa-miR196a</td>
<td>2.07E-21</td>
<td>2.35</td>
<td>Down</td>
<td>0.99</td>
<td>2.33</td>
<td>CCCAACACATGAACTACC</td>
<td>chr12</td>
<td>54,385,548</td>
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<td>54,385,567</td>
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<tr>
<td>hsa-miR32(^c)</td>
<td>2.25E-18</td>
<td>3.12</td>
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<td>1.06</td>
<td>3.22</td>
<td>AAATACACACACTAAATG</td>
<td>chr9</td>
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<td>..</td>
<td>111,808,556</td>
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<tr>
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<td>1.03</td>
<td>2.15</td>
<td>CCCAACACATGAACTACC</td>
<td>chr7</td>
<td>27,209,134</td>
<td>..</td>
<td>27,209,116</td>
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<tr>
<td>hsa-miR190b</td>
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<td>1.95</td>
<td>Down</td>
<td>1.01</td>
<td>1.96</td>
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<td>chr1</td>
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<td>0.42</td>
<td>GCTGAATCCTGCAAATG</td>
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<td>hsa-miR72b</td>
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<td>2.99</td>
<td>Up</td>
<td>1.44</td>
<td>0.48</td>
<td>GCQGAACATTAGCACTG</td>
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<td>2.25</td>
<td>Up</td>
<td>1.23</td>
<td>0.55</td>
<td>GCATATAAGTAATGAGAAGG</td>
<td>chr5</td>
<td>179,442,381</td>
<td>..</td>
<td>179,442,362</td>
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<tr>
<td>hsa-miR62-5p</td>
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<td>2.16</td>
<td>Up</td>
<td>1.18</td>
<td>0.55</td>
<td>ACTCAACATCAGTTG</td>
<td>chrX</td>
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<td>49,660,339</td>
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<td>hsa-miR126</td>
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<td>Up</td>
<td>4.06</td>
<td>0.26</td>
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<td>chr9</td>
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<tr>
<td>hsa-miR148b</td>
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<td>1.12</td>
<td>0.59</td>
<td>ACAAATCTCTGTTAGAC</td>
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<td>Up</td>
<td>1.29</td>
<td>0.58</td>
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<td>Up</td>
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<td>0.67</td>
<td>CAGAACTCTCTGTCAGG</td>
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<td>1.34</td>
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<td>179,442,339</td>
<td>..</td>
<td>179,442,322</td>
</tr>
</tbody>
</table>

\(^{ac}\)Selected test: \(t\) test unpaired; \(P\) value computation: asymptotic.

\(^{b}\)Multiple testing correction: Benjamini–Hochberg; Corrected \(P\) cutoff = 0.05.

\(^{c}\)Fold 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.

### 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 clearly related to the UM-IGHV phenotype (Supplementary Fig. S4A) and its
Table 2. Differentially expressed microRNAs between M-CLL and UM-CLL

<table>
<thead>
<tr>
<th>microRNA</th>
<th>All ( p_{adj} )</th>
<th>Corrected ( P_{adj} )</th>
<th>Fold change</th>
<th>Regulation</th>
<th>CLL [UM] expression</th>
<th>stop</th>
<th>chr Start Stop</th>
<th>miRbase accession</th>
<th>active_sequence chr Start Stop</th>
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<th>MIMAT0000681</th>
<th>MIMAT0003294</th>
<th>MIMAT0000069</th>
<th>MIMAT0004692</th>
<th>MIMAT0000763</th>
<th>MIMAT0004780</th>
<th>MIMAT0004552</th>
<th>MIMAT0000279</th>
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<th>MIMAT0000256</th>
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<tr>
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<td>10.35</td>
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<tr>
<td>hsa-miR625</td>
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<td>1.95</td>
<td>Down</td>
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<td>20.20</td>
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<td>0.72</td>
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<td>0.68</td>
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<td>7.97</td>
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<td>3.27</td>
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</table>

**a** 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 \( \leq 0.05 \).
**e** Fold change cutoff: 1.5.

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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− 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 downregulation 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 (Fig. 3). Among the 14 miRNAs in Table 2, nine showed significant association with PFS (Fig. 3). In particular, CLL cases with miR29c’, miR352-3p, miR146b-5p, miR139-3p, miR222, and miR29c exceeding the median values showed a reduced risk of disease progression, whereas higher expression of miR338-3p, 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; $P = 0.002$) and miR532-3p (HR, 0.46; 95% CI, 0.25–0.82; $P = 0.008$), 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 and UM-CLL cases and (ii) the utilization of a stereotyped IGHV/IGLH 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

<table>
<thead>
<tr>
<th>miRNA</th>
<th>FISH class</th>
<th>Average expression in FISH class</th>
<th>Average expression in all other CLLs</th>
<th>Fold change</th>
<th>Regulation in FISH class</th>
<th>Corrected P&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>active_sequence</th>
<th>chr</th>
<th>Start</th>
<th>Stop</th>
<th>miRbase accession no.</th>
</tr>
</thead>
<tbody>
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<td>4.13</td>
<td>1.52</td>
<td>2.72</td>
<td>Up</td>
<td>6.40E-03</td>
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<td>chr19</td>
<td>46,522,223</td>
<td>46,522,240</td>
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<tr>
<td>hsa-miR338-3p</td>
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<td>4.59</td>
<td>0.98</td>
<td>4.70</td>
<td>Up</td>
<td>9.00E-03</td>
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<td>chr17</td>
<td>79,099,745</td>
<td>79,099,726</td>
<td>MIMAT000763</td>
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<td>47.65</td>
<td>0.16</td>
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<td>2,155,279</td>
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<td>3,439.14</td>
<td>1.54</td>
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<td>4.70E-06</td>
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<sup>a</sup>Selected test: t test unpaired; P value computation: asymptotic.

<sup>b</sup>Multiple 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, 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, 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 to be consistently upregulated in CLL cells by microarray experiments (4, 9, 10, 47, 48). It is also of note that miRNA-29c was identified as an independent predictor of favorable outcome (40, 47) and is in agreement with previous reports (41, 42). The high expression of miR29c, miR338-3p, miR16-5p, 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 analysis, 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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References

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