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

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Abstract

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.

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.

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.

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.*

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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 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). In

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.

addition, expression of certain miRNAs by leukemic cells can provide useful information of prognostic relevance (4).

miRNAs 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 of relevance for disease onset and progression. Importantly, recent studies have suggested the possibility of establishing miRNA-based therapeutics, either by restoring tumor-suppressive miRNAs or by inhibiting miRNAs with pathogenic functions (5).

Aberrantly expressed miRNAs in CLL have been reported by various expression profile studies (6–10), which produced 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-experienced B cells from tonsils and peripheral blood and marginal 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 opportunities for investigating miRNA-based prognostic stratification and therapeutic approaches 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 either 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 $IgD^{bright}CD27^-$ B cells (naïve B cells), $IgD^{low}CD27^+$ 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 ($IgD^{bright}IgM^{bright}CD38^-CD27^-$), marginal zone–like B cells ($IgD^{low}IgM^{bright}$ $CD38^-CD27^+$), 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). $P \leq 0.01$ 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 $\log_2$ 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.

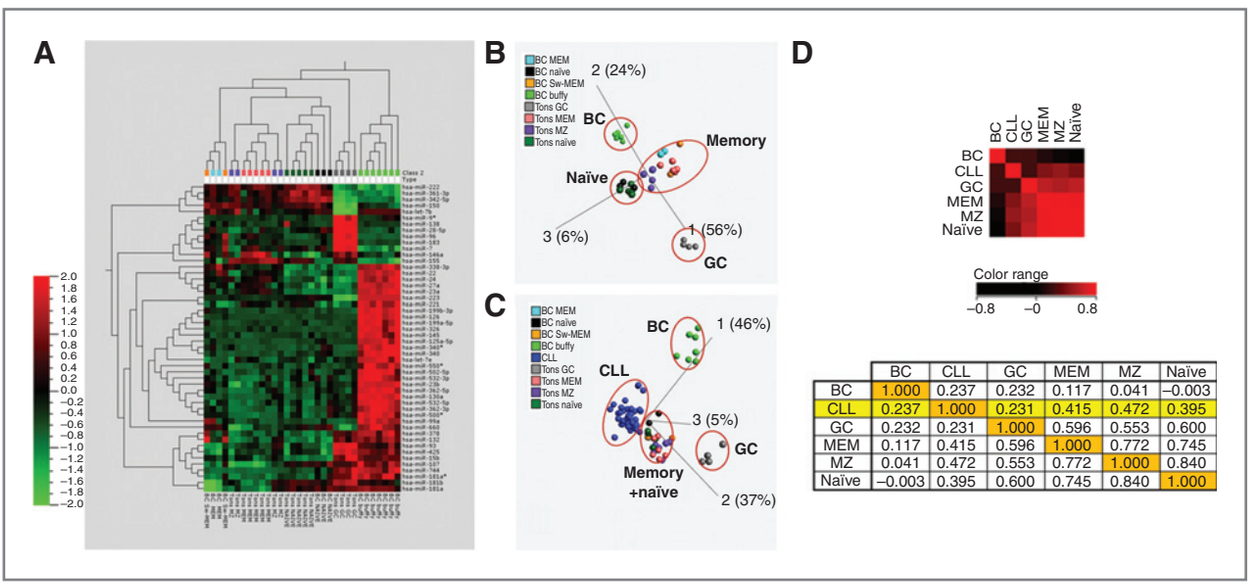
Peripheral blood B cells included 7 samples of buffy coat, 3 samples of naïve B cells, and 2 samples each of IgM-

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 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* (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



	BC	CLL	GC	MEM	MZ	Naïve
BC	1.000	0.237	0.232	0.117	0.041	−0.003
CLL	0.237	1.000	0.231	0.415	0.472	0.395
GC	0.232	0.231	1.000	0.596	0.553	0.600
MEM	0.117	0.415	0.596	1.000	0.772	0.745
MZ	0.041	0.472	0.553	0.772	1.000	0.840
Naïve	−0.003	0.395	0.600	0.745	0.840	1.000

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

miRNA	Corrected $P^{a,b}$	Fold change[c]	Regulation in CLL	CLL expression	Memory B-cell expression	active_sequence	chr	Start		Stop	miRbase accession
hsa-miR193b	4.32E−55	6.49	Down	1.05	6.83	AGCGGGACTTTGAGGG	chr16	14,397,881	..	14,397,895	MIMAT0002819
hsa-miR365	2.51E−36	4.44	Down	0.81	3.60	ATAAGGATTTTTAGGGGCATTA	chr16	14,403,197	..	14,403,218	MIMAT0000710
hsa-miR33b*	7.10E−31	5.92	Down	0.87	5.15	GGGCTGCACTGCCG	chr17	17,717,224	..	17,717,212	MIMAT0004811
hsa-miR196a	2.07E−21	2.35	Down	0.99	2.33	CCCAACAACATGAAACTACC	chr12	54,385,548	..	54,385,567	MIMAT0000226
hsa-miR32*	2.25E−18	3.12	Down	1.06	3.32	AAATATCACACACACTAAATTG	chr9	111,808,576	..	111,808,556	MIMAT0004505
hsa-miR196b	7.85E−16	2.10	Down	1.03	2.15	CCCAACAACAGGAAACTACC	chr7	27,209,134	..	27,209,116	MIMAT0001080
hsa-miR190b	1.34E−13	1.95	Down	1.01	1.96	AACCCAATATCAAACATATCA	chr1	154,166,171	..	154,166,152	MIMAT0004929
hsa-miR23b	4.72E−20	3.44	Up	1.44	0.42	GGTAATCCCTGGCAATG	chr9	97,847,552	..	97,847,567	MIMAT0000418
hsa-miR532-3p	1.46E−18	2.56	Up	1.41	0.55	TGCAAGCCTTGGGTG	chrX	49,767,818	..	49,767,831	MIMAT0004780
hsa-miR27b	1.64E−18	2.99	Up	1.44	0.48	GCAGAACTTAGCCACTGT	chr9	97,847,791	..	97,847,807	MIMAT0000419
hsa-miR340*	2.46E−18	2.25	Up	1.23	0.55	GCTATAAAGTAACTGAGACGG	chr5	179,442,381	..	179,442,362	MIMAT0000750
hsa-miR362-5p	2.41E−16	2.16	Up	1.18	0.55	ACTCACACCTAGGTTCC	chrX	49,660,323	..	49,660,339	MIMAT0000705
hsa-miR126	2.45E−16	15.54	Up	4.06	0.26	CGCATTATTACTCACGGT	chr9	139,565,110	..	139,565,126	MIMAT0000445
hsa-miR148b	3.57E−16	1.88	Up	1.12	0.59	ACAAAGTTCTGTGATGCAC	chr12	54,731,066	..	54,731,083	MIMAT0000759
hsa-miR502-3p	7.28E−16	2.23	Up	1.29	0.58	TGAATCCTTGCCCAGG	chrX	49,779,264	..	49,779,278	MIMAT0004775
hsa-miR103	1.39E−15	1.83	Up	1.15	0.63	TCATAGCCCTGTACAATG	chr5	167,987,970	..	167,987,953	MIMAT0000101
hsa-miR532-5p	1.53E−15	2.39	Up	1.42	0.59	ACGGTCCTACACTCAAG	chrX	49,654,518	..	49,654,534	MIMAT0002888
hsa-miR130a	8.44E−15	9.79	Up	3.05	0.31	ATGCCCTTTTAACATTGCA	chr11	57,408,729	..	57,408,746	MIMAT0000425
hsa-miR361-5p	8.44E−15	1.94	Up	1.23	0.63	GTACCCCTGGAGATTC	chrX	85,045,323	..	85,045,308	MIMAT0000703
hsa-miR26a	1.91E−14	1.81	Up	1.23	0.68	AGCCTATCCTGGATT	chr3	38,010,911	..	38,010,925	MIMAT0000082
hsa-miR330-3p	4.32E−14	1.89	Up	1.07	0.57	TCTCTGCAGGCCGTG	chr19	46,142,330	..	46,142,316	MIMAT0000751
hsa-miR500*	1.35E−12	1.82	Up	1.23	0.67	CAGAATCCTTGCCCAGGT	chrX	49,659,834	..	49,659,851	MIMAT0002871
hsa-miR744	2.06E−12	2.28	Up	1.34	0.59	TGCTGTTAGCCCTA	chr17	11,985,235	..	11,985,247	MIMAT0004945
hsa-miR340	5.35E−12	3.23	Up	1.64	0.51	AATCAGTCTCATTGCTTTA	chr5	179,442,339	..	179,442,322	MIMAT0004692

[a]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.

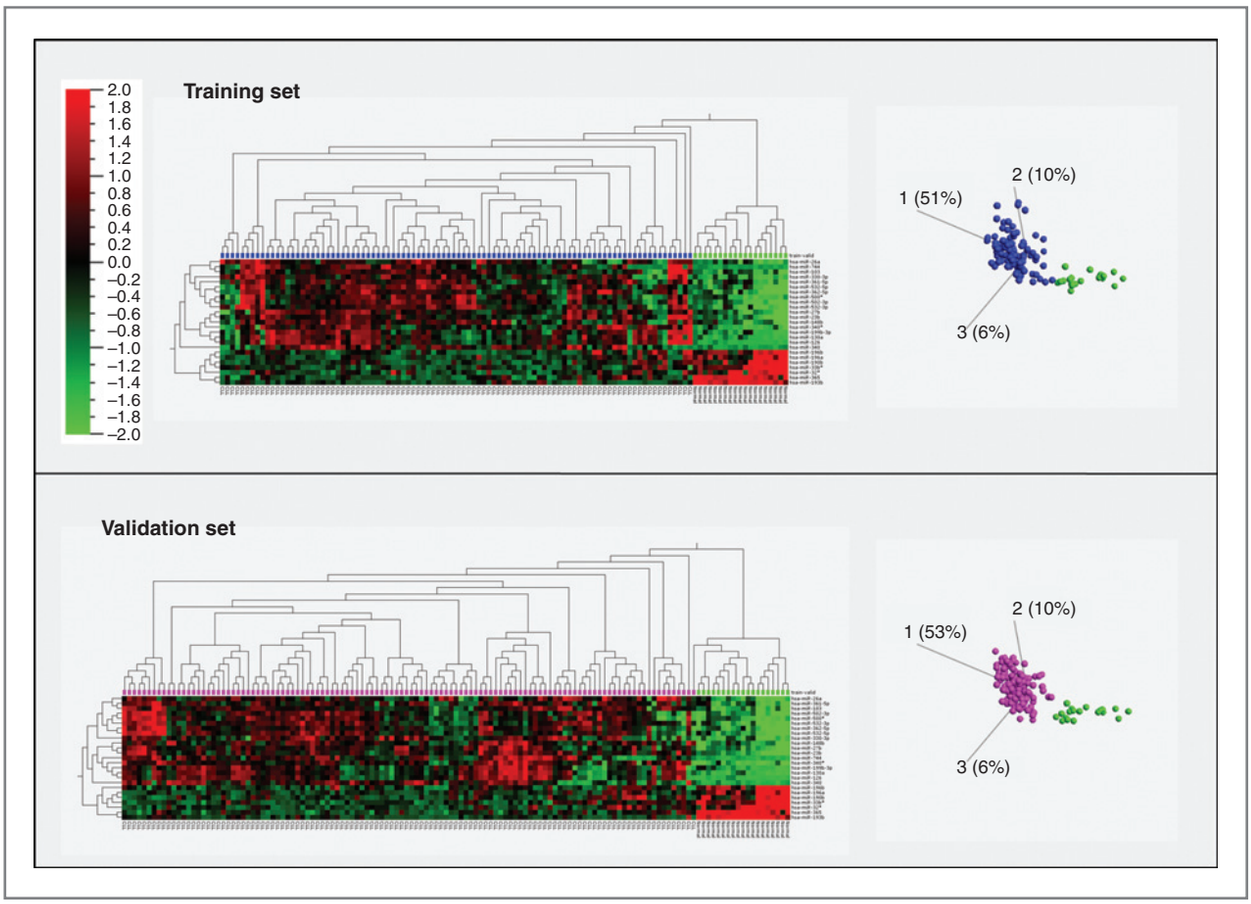


Figure 2. miRNA expression in CLLs versus normal B cells. Unsupervised cluster and PCA analyses based on the miRNAs are shown in Table 1. Top, the analyses based on the CLL training set of samples; bottom, the analyses of the independent validation set. Both sets exhibit an excellent separation between 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].

shown). 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

miRNA	All[a]	13q− [b]	Corrected *P*[c,d]	Fold change[e]	Regulation UM/M	CLL [M] expression	CLL [UM] expression	active_sequence	chr	Start	Stop	miRbase accession
hsa-miR146b-5p	1	1	1.41E−07	2.93	Down	39.59	13.50	AGCCTATGGAATTCAGTTC	chr10	104,186,270	104,186,288	MIMAT0002809
hsa-miR29c*	1	1	1.41E−07	3.12	Down	14.02	4.49	GAACACCAGGAGAAATCGGT	chr1	207,975,233	207,975,215	MIMAT0004673
hsa-miR29c	1	1	1.31E−04	1.89	Down	1,542.98	817.74	TAACCGATTTCAAATGGTGCTA	chr1	207,975,271	207,975,251	MIMAT0000681
hsa-miR625	1	1	5.81E−04	1.95	Down	20.20	10.35	GGACTATAGAACTTTCCCC	chr14	65,937,837	65,937,854	MIMAT0003294
hsa-miR16	1		3.65E−03	1.37	Up	2,557.27	3,504.23	CGCCAATATTTACGTGCTG	chr3	160,122,545	160,122,563	MIMAT0000069
hsa-miR340	1		3.90E−03	2.47	Up	0.39	1.01	AATCAGTCTCATTGCTTTA	chr5	179,442,339	179,442,322	MIMAT0004692
hsa-miR338-3p	1	1	5.61E−03	2.89	Up	0.72	2.08	CAACAAAATCACTGATGCTGG	chr17	79,099,745	79,099,726	MIMAT0000763
hsa-miR340*	1	1	5.61E−03	1.97	Up	0.68	1.33	GCTATAAAGTAACTGAGACGG	chr5	179,442,381	179,442,362	MIMAT0000750
hsa-miR532-3p	1	1	5.61E−03	1.93	Down	3.16	1.64	TGCAAGCCTTGGGTG	chrX	49,767,818	49,767,831	MIMAT0004780
hsa-miR139-3p	1		5.61E−03	1.93	Down	1.70	0.88	ACTCCAACAGGGCCG	chr11	72,326,171	72,326,158	MIMAT0004552
hsa-miR222	1		1.23E−02	1.59	Down	7.97	5.02	ACCCAGTAGCCAG	chrX	45,606,509	45,606,498	MIMAT0000279
hsa-miR151-5p	1		1.37E−02	1.73	Down	90.05	51.94	ACTAGACTGTGAGCTCC	chr8	141,742,693	141,742,678	MIMAT0004697
hsa-miR574-3p	1		3.57E−02	1.53	Up	3.27	5.00	TGTGGGTGTGTGCATG	chr4	38,869,720	38,869,734	MIMAT0003239
hsa-miR181a	1		4.91E−02	1.75	Down	14.12	8.07	ACTCACCGACAGCGT	chr1	198,828,218	198,828,204	MIMAT0000256

[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− 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*, miR532-3p, 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 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

miRNA	FISH class	Average expression in FISH class	Average expression in all other CLLs	Fold change	Regulation in FISH class	Corrected $P^{a,b}$	active_sequence	chr	Start	Stop	miRbase accession no.
hsa-miR769-5p	11q−	4.13	1.52	2.72	Up	6.40E−03	AGCTCAGAACCCAGAGGTC	chr19	46,522,223	46,522,240	MIMAT0003886
hsa-miR338-3p	11q−	4.59	0.98	4.70	Up	9.00E−03	CAACAAAATCACTGATGCTGG	chr17	79,099,745	79,099,726	MIMAT0000763
hsa-miR155	12+	253.16	546.91	2.16	Down	4.20E−10	ACCCCTATCACGATTAG	chr21	26,946,302	26,946,317	MIMAT0000646
hsa-miR148a	12+	8.13	47.65	5.86	Down	1.60E−09	ACAAAGTTCTGTAGTGCACT	chr7	25,989,603	25,989,585	MIMAT0000243
hsa-miR483-5p	12+	0.87	4.37	5.05	Down	2.30E−05	CTCCCTTCTTTCCTC	chr11	2,155,392	2,155,379	MIMAT0004761
hsa-miR16	13q−	2,226.24	3,439.14	1.54	Down	4.70E−06	CGCCAATATTTACGTGCTG	chr3	160,122,545	160,122,563	MIMAT0000069
hsa-miR146b-5p	13q−	41.92	19.81	2.12	Up	2.40E−05	AGCCTATGGAATTCAGTTC	chr10	104,186,270	104,186,288	MIMAT0002809
hsa-miR363	13q−	6.28	15.56	2.48	Down	2.90E−04	TACAGATGGATACCGTGCA	chrX	133,303,478	133,303,461	MIMAT0000707
hsa-miR148a	13q−	56.93	27.68	2.06	Up	8.50E−04	ACAAAGTTCTGTAGTGCACT	chr7	25,989,603	25,989,585	MIMAT0000243
hsa-miR34a	17p−	3.43	47.81	13.93	Down	6.60E−06	ACAACCAGCTAAGACACTGC	chr1	9,211,769	9,211,751	MIMAT0000255
hsa-miR150	17p−	5,471.01	10,831.80	1.98	Down	8.50E−04	CACTGGTACAAGGGTTGG	chr19	50,004,078	50,004,062	MIMAT0000451
hsa-miR33b*	17p−	3.24	0.51	6.30	Up	3.20E−03	GGGCTGCACTGCCG	chr17	17,717,224	17,717,212	MIMAT0004811
hsa-miR96	17p−	1.21	0.22	5.51	Up	3.40E−03	AGCAAAAATGTGCTAGTGCCAA	chr7	129,414,562	129,414,542	MIMAT0000095
hsa-miR21*	17p−	15.60	5.97	2.61	Up	5.10E−03	ACAGCCCATCGACTG	chr17	57,918,679	57,918,692	MIMAT0004494
hsa-miR425*	FISH neg	1.98	7.11	3.57	Down	1.60E−09	GGGCGGACACGAC	chr3	49,057,656	49,057,645	MIMAT0001343
hsa-miR1237	FISH neg	1.38	3.67	2.63	Down	2.40E−05	CTGGGGGACGGAG	chr11	64,136,164	64,136,175	MIMAT0005592
hsa-miR484	FISH neg	1.02	2.22	2.21	Down	9.60E−05	ATCGGGAGGGGACTGA	chr16	15,737,165	15,737,179	MIMAT0002174

[a]Selected test: *t* test unpaired; P value computation: asymptotic.

[b]Multiple testing correction: Benjamini–Hochberg; Corrected *P* cutoff = 0.05.

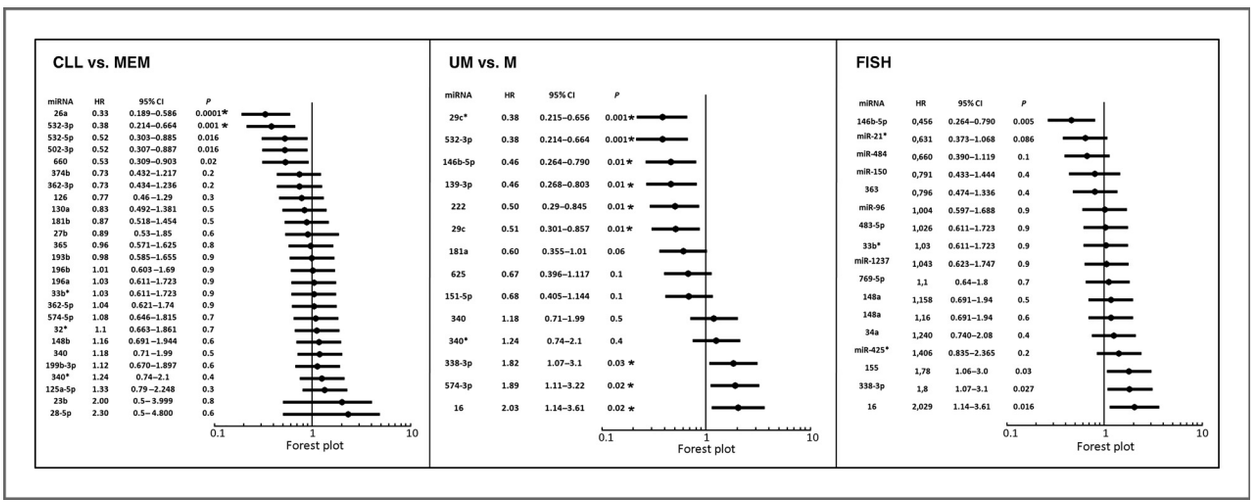


Figure 3. Cox univariate analyses of miRNAs in PFS. Forest plots depicting PFS, defined as the time from diagnosis to first treatment, of subgroups of patients stratified according to the lists of miRNAs shown in Tables 1 to 3. HR was calculated: HR lower or higher than 1 indicates a lower and a higher risk of progression in relation to the increase in miRNA expression, respectively. The bars represent the 95% CIs. The asterisk in *P* value column indicates miRNA that retained significance after multiple hypothesis testing adjustment.

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 downregulation 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 pathogenic 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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References

1. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med 2005;352:804–15.
2. Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med 2000;343:1910–6.
3. Gaidano G, Foa R, Dalla-Favera R. Molecular pathogenesis of chronic lymphocytic leukemia. J Clin Invest 2012;122:3432–8.
4. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 2005;353:1793–801.
5. Joshi D, Gosh K, Vundinti BR. MicroRNAs in hematological malignancies: a novel approach to targeted therapy. Hematology 2012;17:170–5.
6. Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci U S A 2004;101:11755–60.
7. Fulci V, Chiaretti S, Goldoni M, Azzalin G, Carucci N, Tavolaro S, et al. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. Blood 2007;109:4944–51.
8. Lawrie CH, Saunders NJ, Soneji S, Palazzo S, Dunlop HM, Cooper CD, et al. MicroRNA expression in lymphocyte development and malignancy. Leukemia 2008;22:1440–6.
9. Lawrie CH, Ballabio E, Dyar OJ, Jones M, Ventura R, Chi J, et al. MicroRNA expression in chronic lymphocytic leukaemia. Br J Haematol 2009;147:398–402.
10. Li S, Moffett HF, Lu J, Werner L, Zhang H, Ritz J, et al. MicroRNA expression profiling identifies activated B cell status in chronic lymphocytic leukemia cells. PLoS One 2011;6:e16956.
11. Cutrona G, Colombo M, Matis S, Fabbi M, Spriano M, Callea V, et al. Clonal heterogeneity in chronic lymphocytic leukemia cells: superior response to surface IgM cross-linking in CD38, ZAP-70-positive cells. Haematologica 2008;93:413–22.
12. Morabito F, De Filippi R, Laurenti L, Zirlik K, Recchia AG, Gentile M, et al. The cumulative amount of serum-free light chain is a strong prognosticator in chronic lymphocytic leukemia. Blood 2011;118:6353–61.
13. Zenz T, Mohr J, Eldering E, Kater AP, Buhler A, Kienle D, et al. miR-34a as part of the resistance network in chronic lymphocytic leukemia. Blood 2009;113:3801–8.
14. Zenz T, Habe S, Denzel T, Mohr J, Winkler D, Buhler A, et al. Detailed analysis of p53 pathway defects in fludarabine-refractory chronic lymphocytic leukemia (CLL): dissecting the contribution of 17p deletion, TP53 mutation, p53-p21 dysfunction, and miR34a in a prospective clinical trial. Blood 2009;114:2589–97.
15. Mosca L, Fabris S, Lionetti M, Todoerti K, Agnelli L, Morabito F, et al. Integrative genomics analyses reveal molecularly distinct subgroups of B-cell chronic lymphocytic leukemia patients with 13q14 deletion. Clin Cancer Res 2010;16:5641–53.
16. Dono M, Zupo S, Leanza N, Melioli G, Fogli M, Melagrana A, et al. Heterogeneity of tonsillar subepithelial B lymphocytes, the splenic marginal zone equivalents. J Immunol 2000;164:5596–604.
17. Basso K, Sumazin P, Morozov P, Schneider C, Maute RL, Kitagawa Y, et al. Identification of the human mature B cell miRNome. Immunity 2009;30:744–52.
18. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med 2001;194:1625–38.
19. Seifert M, Sellmann L, Bloehdorn J, Wein F, Stilgenbauer S, Durig J, et al. Cellular origin and pathophysiology of chronic lymphocytic leukemia. J Exp Med 2012;209:2183–98.
20. Kikushige Y, Ishikawa F, Miyamoto T, Shima T, Urata S, Yoshimoto G, et al. Self-renewing hematopoietic stem cell is the primary target in pathogenesis of human chronic lymphocytic leukemia. Cancer Cell 2011;20:246–59.
21. Gross E, Quillet-Mary A, Ysebaert L, Laurent G, Fournie JJ. Cancer stem cells of differentiated B-cell malignancies: models and consequences. Cancers (Basel) 2011;3:1566–79.
22. Chiorazzi N, Ferrarini M. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. Blood 2011;117:1781–91.
23. Mraz M, Malinova K, Kotaskova J, Pavlova S, Tichy B, Malcikova J, et al. miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. Leukemia 2009;23:1159–63.
24. Rigolin GM, Saccenti E, Rizzotto L, Ferracin M, Martinelli S, Formigaro L, et al. Genetic subclonal complexity and miR125a-5p down-regulation identify a subset of patients with inferior outcome in low-risk CLL patients. Oncotarget 2014;5:140–9.
25. Ferrajoli A, Shanafelt TD, Ivan C, Shimizu M, Rabe KG, Nouraee N, et al. Prognostic value of miR-155 in individuals with monoclonal B-cell lymphocytosis and patients with B chronic lymphocytic leukemia. Blood 2013;122:1891–9.
26. Dufour A, Palermo G, Zellmeier E, Mellert G, Duchateau-Nguyen G, Schneider S, et al. Inactivation of TP53 correlates with disease progression and low miR-34a expression in previously treated chronic lymphocytic leukemia patients. Blood 2013;121:3650–7.
27. Visone R, Veronese A, Rassenti LZ, Balatti V, Pearl DK, Acunzo M, et al. miR-181b is a biomarker of disease progression in chronic lymphocytic leukemia. Blood 2011;118:3072–9.
28. Rossi S, Shimizu M, Barbarotto E, Nicoloso MS, Dimitri F, Sampath D, et al. microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. Blood 2010;116:945–52.
29. Vargova K, Curik N, Burda P, Basova P, Kulvait V, Pospisil V, et al. MYB transcriptionally regulates the miR-155 host gene in chronic lymphocytic leukemia. Blood 2011;117:3816–25.
30. Mraz M, Dolezalova D, Plevova K, Stano Kozubik K, Mayerova V, Cerna K, et al. MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia. Blood 2012;119:2110–3.
31. Bomben R, Gobessi S, Dal Bo M, Volinia S, Marconi D, Tissino E, et al. The miR-17 approximately 92 family regulates the response to Toll-like receptor 9 triggering of CLL cells with unmutated IGHV genes. Leukemia 2012;26:1584–93.
32. Kovaleva V, Mora R, Park YJ, Plass C, Chiramel AI, Bartenschlager R, et al. miRNA-130a targets ATG2B and DICER1 to inhibit autophagy and trigger killing of chronic lymphocytic leukemia cells. Cancer Res 2012;72:1763–72.
33. Zhu DX, Fan L, Lu RN, Fang C, Shen WY, Zou ZJ, et al. Downregulated Dicer expression predicts poor prognosis in chronic lymphocytic leukemia. Cancer Sci 2012;103:875–81.
34. Rauhala HE, Jalava SE, Isotalo J, Bracken H, Lehmusvaara S, Tammela TL, et al. miR-193b is an epigenetically regulated putative tumor suppressor in prostate cancer. Int J Cancer 2010;127:1363–72.
35. Zhu W, Shan X, Wang T, Shu Y, Liu P. miR-181b modulates multidrug resistance by targeting BCL2 in human cancer cell lines. Int J Cancer 2010;127:2520–9.

36. Lu J, He ML, Wang L, Chen Y, Liu X, Dong Q, et al. MiR-26a inhibits cell growth and tumorigenesis of nasopharyngeal carcinoma through repression of EZH2. Cancer Res 2011;71:225–33.
37. Narducci MG, Arcelli D, Picchio MC, Lazzeri C, Pagani E, Sampogna F, et al. MicroRNA profiling reveals that miR-21, miR486 and miR-214 are upregulated and involved in cell survival in Sezary syndrome. Cell Death Dis 2011;2:e151.
38. Forrest AR, Kanamori-Katayama M, Tomaru Y, Lassmann T, Ninomiya N, Takahashi Y, et al. Induction of microRNAs, mir-155, mir-222, mir-424 and mir-503, promotes monocytic differentiation through combinatorial regulation. Leukemia 2010;24:460–6.
39. Sun X, Icli B, Wara AK, Belkin N, He S, Kobzik L, et al. MicroRNA-181b regulates NF-kappaB-mediated vascular inflammation. J Clin Invest 2012;122:1973–90.
40. Pekarsky Y, Santanam U, Cimmino A, Palamarchuk A, Efanov A, Maximov V, et al. Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. Cancer Res 2006;66:11590–3.
41. Gao XN, Lin J, Gao L, Li YH, Wang LL, Yu L. MicroRNA-193b regulates c-Kit proto-oncogene and represses cell proliferation in acute myeloid leukemia. Leuk Res 2011;35:1226–32.
42. Messmer BT, Messmer D, Allen SL, Kolitz JE, Kudalkar P, Cesar D, et al. *In vivo* measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. J Clin Invest 2005;115:755–64.
43. Hermeking H. The miR-34 family in cancer and apoptosis. Cell Death Differ 2010;17:193–9.
44. Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci U S A 2006;103:7024–9.
45. O'Connell RM, Rao DS, Chaudhuri AA, Boldin MP, Taganov KD, Nicoll J, et al. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. J Exp Med 2008;205:585–94.
46. Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med 2001;194:1639–47.
47. Stamatopoulos B, Meuleman N, Haibe-Kains B, Saussoy P, Van Den Neste E, Michaux L, et al. microRNA-29c and microRNA-223 down-regulation has *in vivo* significance in chronic lymphocytic leukemia and improves disease risk stratification. Blood 2009;113:5237–45.
48. Zhao JJ, Lin J, Lwin T, Yang H, Guo J, Kong W, et al. microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. Blood 2010;115:2630–9.
49. Pekarsky Y, Croce CM. Is miR-29 an oncogene or tumor suppressor in CLL? Oncotarget 2010;1:224–7.
50. Santanam U, Zanesi N, Efanov A, Costinean S, Palamarchuk A, Hagan JP, et al. Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression. Proc Natl Acad Sci U S A 2010;107:12210–5.