MicroRNAome expression in chronic lymphocytic leukemia: comparison with normal B cell subsets and correlations with prognostic and clinical parameters

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RUNNING TITLE: microRNA expression in CLL

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TRANSLATIONAL RELEVANCE

Evidence indicated a role for miR-15/16 in 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 CLLs 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.
ABSTRACT

**Purpose:** Despite its indolent nature, chronic lymphocytic leukemia (CLL) remains an incurable disease. To establish the potential pathogenic role of microRNAs (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 miR-26a, miR-532-3p, miR-146-5p and miR-29c* 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 utilized for developing miRNA-based therapeutic strategies in CLL.
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 towards more advanced stages and may never require therapy or only after years from diagnosis, while 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 based upon 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 addition, expression of certain microRNAs (miRNAs) by leukemic cells can provide useful information of prognostic relevance (4).

MiRNAs are short non-coding RNAs that play a key role in post-transcriptional 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 employed 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 Binet stage A CLL patients 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 (MZ)-like B-cells. Antigen-experienced and MZ-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 CLL patients 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

CLL patients 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-cytometry analysis; (iii) clinical Binet stage B or C; (iv) need of therapy according to NCI guidelines; (v) age >70 years. Diagnosis was confirmed by flow-cytometry analysis, together with the determination of CD38 and ZAP-70 expression and IGHV mutational status. Peripheral blood mononuclear cells from CLL patients were isolated by Ficoll-Hypaque (Seromed, Biochrom KG, Berlin, Germany) 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, Milan, Italy) 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, San José California, USA), CD23-PE (BD Biosciences, BD), and CD5-PC5 (Beckman Coulter Immunotech, Marseille, France). ZAP-70 expression was determined by flow-cytometry with a ZAP-70 FITC (clone 2F3.2, Millipore, Temecula, California, USA) 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 germ-line gene were considered mutated. Deletions at chromosomes 11q23, 13q14 and 17p13 and trisomy 12 were evaluated by fluorescence in situ hybridization (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 RPMI1640 containing 10% fetal bovine serum (FBS) (Invitrogen, Paisley, UK) 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, Vancouver, Canada). The remaining cells were then sorted by fluorescent activated cell sorting (FACS) (FACS Aria, Becton Dickinson, San Jose, CA) 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: fluorescein isothiocyanate (FITC)-labeled polyclonal anti-δ (Dako), Pe-Cy5 or PE-CF594-labeled anti-CD27 mAb (BD), or PC7-labeled anti-CD38 mAb (BD). CD19+ B-cells from buffy coats, depleted of CD38 to exclude plasma cells (called B cells or BC), were further sorted into IgDbrightCD27+ B-cells (Naïve B-cells), IgDlowCD27+ cells (IgM-memory or IgM-mem B-cells), and IgD-CD27+ cells (switched-memory or SM B-cells). Due to the paucity of B-cells, the two samples included in the analyses represent pools of cell subset populations from two and three buffy coats, respectively. Tonsil CD19+ B-cells were separated further based on the expression of IgD versus CD38 molecules. The following non-adjacent gates were drawn to separate naive B-cells (IgDbrightIgMbrightCD38CD27), MZ-like B-cells (IgDlowIgMbrightCD38´CD27+), germinal center (GC) B-
Agilent miRNA microarrays and data analysis

Total RNA fraction was obtained using the Trizol reagent (Life Technologies, Invitrogen, Carlsbad, California, USA). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Low quality RNA (integrity < 7) were excluded from microarray analyses. RNAs were hybridized on an Agilent Human miRNA microarray (G4470B, Agilent Technologies, Palo Alto, CA) 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, Lund, Sweden). 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 employed 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’s correction using the GraphPad Prism software (GraphPad Software Inc., CA, USA). The correlation coefficients between classes were calculated using Pearson’s correlation coefficient (GeneSpring GX software, Agilent Technologies, Palo Alto, CA, USA 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 hazard ratio (HR) and 95% confidence interval. A value of 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 seventeen Binet stage A CLL patients were investigated. Their specific features are summarized in Supplementary Table 1. 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 CD-38 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 (MM), and Sezary syndrome (SS) samples were investigated. A cluster analysis was performed using expressed miRNAs (average log2 expression >0 in at least one class), selected as differentially expressed in a multiclass ANOVA analysis (p-value<0.01). The results of the analysis, shown as heat map and principal component analysis (PCA) in Supplementary Figure 2, 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 the Materials and Methods section. They included peripheral blood CD19^CD38^- B-cells (B-cells depleted of plasma cells) as well as naïve, IgM-memory and switched-memory (SM) B-cells. The following B-cell subpopulations from tonsils were used: germinal center (GC), Naïve, MZ-like, and SM memory B-cells. Supplementary Figure 1 describes the FACS sorting strategy used to purify these cell subpopulations.

Peripheral blood B-cells included 7 samples of BC, 3 samples of naïve B-cells, and 2 samples each of IgM-Memory and SM B-cells, with each sample representing a pool derived from two or three buffy coats. Tonsil samples included 4 GC, 5 naïve, 4 MZ-like cells, and 5 SM B-cells, each sample being a pool of cells obtained from two or three tonsils. Based on PCA and unsupervised cluster analysis, a set of 50 miRNAs produced the best discrimination between the different subsets of normal B-cells (Figure 1A-B). The patterns of miRNA expression of IgM-memory and SM B-cells from peripheral blood and of MZ-like and SM 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 while tonsil GC B-cells and peripheral blood BC 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 CLLs were more similar to tonsil MZ-like B-cells and to the group of antigen-experienced B-cells including peripheral blood IgM-memory and SM B-cells and tonsil SM B-cells. Naïve B-cells from peripheral blood and tonsils also were more similar to CLL cells than tonsil GC B-cells or peripheral blood BC. (Figure 1C). In a second approach, a correlation analysis using the Pearson correlation coefficient (CC), 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 MZ-like B-cells (CC= 0.472) and SM memory* (CC = 0.415) B-cells. Naïve B-cells exhibited a moderate correlation (CC= 0.395), while markedly lower values were obtained for peripheral blood BC (CC= 0.237) and tonsil GC B-cells (CC= 0.231) (Figure 1D).
Differentially expressed miRNAs possibly involved in CLL pathogenesis

We next compared the CLL miRNA expression profile to that of normal B-cells including tonsil MZ-like (5 samples), SM B-cells (5 samples), peripheral blood IgM-memory (5 samples) and SM B-cells (5 samples). Given the similarities of miRNA profile of these normal subpopulations (collectively, antigen experienced B-cells plus MZ-like B-cells), these could be combined for comparisons with CLL cells and will be denominated henceforth as the “comparator” cells. When the miRNA expression profiles of the cells from an unselected cohort of 99 CLL cases (training set) was compared to the comparator cells, 106 miRNAs differentially expressed between the two groups were identified (p-value < 0.01; Supplementary Table 2). 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 sub-group of CLLs (Table 1 and data not 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 + MZ-like) B-cells. (Figure 2); notably, it maintained an excellent discriminating capacity when applied to a second independent CLL group (validation set, n = 114)(Figure 2). Clinical and bio-molecular characteristics between CLL training and validation sets did not differ significantly (Supplementary Table 1).

Some of the discriminating miRNAs, miR-125a-5p, miR-130a, miR-365, miR-193b and miR-26a were further validated on a third group of independent unselected CLLs (n = 20) by using a quantitative real-time PCR (RT-qPCR). All the analyses confirmed to a great extent the microarray results (Supplementary Figure 3); only miR-181b, which showed a significant down-regulation 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 log2 expression > 0.0 in at least one of the classes, fold change > 1.5, and 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 two 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, miR-29c* and miR-146b-5p possessed the most significant p-values. The low miR-29c* expression was the feature most clearly related to the UM-IGHV phenotype (Supplementary Figure 4A) and its expression per se provided a tool to distinguish between M-CLL and UM-CLL (Supplementary Figure 4B). Notably, the miR-29c/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 Figure 4C). This finding may explain why miR-29c/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 miR-146b-5p, miR-29c, miR-625 and miR-532-3p, were further analyzed with RT-qPCR providing good and significant correlation coefficients with microarray analysis (Supplementary Figure 5).

**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 Figure 6 visually represent the average pattern of expression of these miRNAs in each class versus the other CLL classes (right panel) or versus normal “comparator” cells (left panel). The clearest results included the down-regulation of miR-34a and the up-regulation of miR-96 and miR-21* in 17p- CLLs; the up-regulation of miR-338-3p in the 11q- class; the down-regulation of miR-148a, miR-21*, miR-155 and miR-483-5p in trisomy 12 cases; and the down-regulation of miR-16 in 13q- cases, more evident in patients with biallelic deletion.

Validation based on RT-qPCR was performed only for miR-146b-5p (see Supplementary Figure 5) because the down-regulation of miR-34a in the 17p- CLL cases (13, 14) and the down-regulation of miR-16 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 progression free survival (PFS) of miRNA lists reported in Tables 1, 2 and 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 five miRNAs (miR-26a, miR-532-3p, miR-532-5p, miR-502-3p and miR-660) was significantly associated with a shorter PFS (Figure 3). Among the 14 miRNAs in Table 2, nine showed significant association with PFS (Figure 3). In particular, CLL cases with miR-29c*, miR-532-3p, miR-146b-5p, miR-139-3p, miR-222 and miR-29c exceeding the median values, showed a reduced risk of disease progression; while higher expression of miR-338-3p, miR-574-3p, miR-16 exhibited an unfavorable clinical outcome. Finally, among the 17 miRNAs in Table 3, four were significantly associated with PFS (Figure 3): high expression of miR-146b-5p appeared to have a protective effect from disease progression, whereas high expression of miR-155, miR-338-3p and miR-16 had an opposite effect. Overall, eleven out of the eighteen miRNAs associated with PFS retained significance after multiple test adjustments (Figure 3): after data adjustment for confounders CD38 and ZAP-70 expression, FISH data, and IGHV mutational status, only two miRNAs, miR-26a (H.R. 0.41, 95% C.I. 0.23-0.73, P=.002) and miR-532-3p (H.R. 0.46, 95% C.I. 0.25-0.82, P=.008), remained significantly associated with PFS.
DISCUSSION

In this study, cells from a large cohort of Binet A stage CLL patients were investigated for miRNA expression profiling and the data compared to 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 SM memory B-cells from peripheral blood and SM B-cells from tonsils. In addition, tonsil MZ-like B-cells exhibited a miRNA signature very similar to that of tonsil SM B-cells and of CLL cells, a finding not unexpected considering that MZ-like B-cells are mostly antigen experienced B-cells and home in the same anatomical area as SM B-cells (16). Other B-cell subsets, like circulating BC and of GC 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 et al 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: (1) the presence of somatic IGHV hypermutation in M-CLL cases, and (2) the utilization of a stereotyped IGHV/IGHL gene repertoire by CLL compared to 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 et al. found that memory B-cells were the most similar to CLL cells using gene expression profiling technologies (19). However, evidence for a heterogeneous origin of CLL cells from CD5⁻ B-cell subpopulations (CD27⁻ or CD27⁺) was recently presented (18). Yet, previous studies indicated that normal CD5⁺ B-cells exhibit rather unique miRNA and gene expression profiles compared to CLL (6, 19).

The above data provide clues regarding the nature of the CLL cell of origin. However, a note of caution should be 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 which used unsorted circulating B-cells as comparators (7, 9, 10); in particular, up-regulation of miR-155 and miR-150 (7, 9, 10) was observed as well as the down-regulation of miR-181b (10), miR-222 and miR-92 (7). Our results established that miR-34a is generally up-regulated in CLL, as reported by Li et al. (10), but also confirmed that it is strongly down-regulated in CLL cases with deletion at 17p (14, 23). However, other miRNAs were found to be deregulated using the specific approach reported herein, including miRNAs miR-193b, miR-33b*, and miR-196 among the down-regulated miRNAs and miR-23b, miR-26a and miR-532 (5p and 3p) among the up-regulated 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 down-regulation of miR-34a and the up-regulation of miR-155 (25, 29) and miR-21 in 17p- cases (28). The down-regulation of miR-181b 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 miR-650 or miR-17-92 family in CLL.

Based on 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 down-regulation of miR-181 and miR-193b and the up-regulation of miR-26a, miR-125a-5p, miR-130a and miR-155 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 miR-181b (35, 39, 40), c-KIT, MCL1, cyclin D1, and ETS1 for miR-193b (41). The miR-130a 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 miR-29c*, miR-29c, miR-26a, miR-532-3p, miR-146b-5p, miR-139-3p, miR-222, miR-338-3p, miR-574-3p, miR-155, and miR-16, 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 up-regulation of miR-34a 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 the patient’s prognosis poor. A similar scenario may also be claimed for miR-130a 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 miR-130a exhibits a reduced expression in CLL cells when CD19+ peripheral blood cells are used as comparators (32), miR-130a 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 miR-16 expression was significantly associated with bi-allelic 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 miR-34a was closely related to the presence of 17p deletion (14, 23). Because miR-34a expression is under the transcriptional control of p53 (see (43) for a recent review), this result suggests that deletion at 17p, where the TP53 gene is located, may influence downstream TP53 targets, including miR-34a (14, 23). Also of note, it is the observed down-regulation of miR-155 in CLLs with trisomy 12, in agreement with a previous report (9). However, this may represent an odd evidence, given that miR-155 is one of the most well-established oncomirs in hematologic and solid tumors (44, 45) and is found up-regulated 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, miR-29c* and its partner miR-29c could discriminate cases from the two groups. Both miRNAs were down-regulated in the
UM-CLL poor prognosis group. It has been reported that members of the miR-29 family have the ability of down-modulating 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 up-regulated in CLL cells by Lawrey et al. who did not, however, subdivide their cases into M-and U-CLL (9). Indeed, in our study miR-29 (mainly miR-29c and miR-29c*) exhibited a weak up-regulation in M-CLLs compared to normal B-cells. Overall, these observations suggest that miR-29 oncogenic activity could depend upon cellular background (49): the up-regulation 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 down-regulated.

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 miR-29c*, miR-532-3p, miR-146b-5p, miR-139-3p, miR-222 and miR-29c was associated with a reduced risk of disease progression, while the opposite was true for miR-338-3p, miR-574-3p and miR-16. Likewise, among the miRNAs associated with cytogenetic abnormalities, high levels of miR-146b-5p appeared to have a protective effect from disease progression while the opposite was observed for miR-155, miR-338-3p and miR-16. Some of these miRNA (miR-29c, miR-146, miRNA-222 and miR-155) were included in an earlier miRNA-based signature associated with PFS prediction (4). However, among all the above, only miR-26a and miR-532-3p (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 INTERESTS

The authors declare no potential conflicts of interests

AUTHORS’ CONTRIBUTIONS

MN, AN, MF designed the study and wrote the manuscript; AC, GAC, SS, GR, PT, FM provided samples and participated to the final design of the study; GC, MC, SM, DR isolated and characterized normal B cell populations; SF, LD’A, ES, ML, MG, AGR, SB prepared RNA samples, CB, MF, BZ, LA performed microarray experiments and analyses. All authors critically reviewed and edited the manuscript.

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REFERENCES


LEGENDS TO FIGURES

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-miRNAs expression profile that best discriminate the normal B-cell subsets. (B) similar to the cluster analysis, a Principal component analysis (PCA) shows that antigen-experienced B cells, inclusive of peripheral blood IgM-memory and SM B-cells and of tonsil SM B cell, MZ cells and Naïve B-cells are close in a three-dimensional space of similarity, while BC and 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 Pearson’s correlation coefficient. BC = buffy coat; Tons = tonsil; BC buffy = peripheral blood CD19+ B lymphocytes; MEM = memory B-cells; NAÏVE = naïve B-cells; Sw-MEM = switched memory B-cells; MZ = marginal zone B-cells, analogous to SE, sub-epithelial, B-cells; GC = germinal center B-cells.

Figure 2. miRNA expression in CLLs versus normal B-cells. Unsupervised cluster and PCA analyses based on the miRNAs are shown in Table 1. The upper panel shows the analyses based on the CLL training set of samples; the lower panel shows the analyses of the independent validation set. Both sets exhibit an excellent separation between CLL (blue label in upper panel; cyan label in lower panel) and “comparator” B-cells (PB SM B-cells + PB IgM-memory B-cells + tonsil SM B-cells + tonsil MZ-like B-cells) (green label in both panels).

Figure 3. Cox univariate analyses of miRNAs in progression-free survival. 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 1-3. Hazard ratio (H.R.) was calculated: H.R. 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% confidence intervals. The asterisk in p-value column indicates miRNA that retained significance after multiple hypothesis testing adjustment.
Table 1. MicroRNAs commonly dysregulated in CLL versus normal “comparator” B cells

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Corrected p-value</th>
<th>Fold change</th>
<th>Regulation in CLL</th>
<th>CLL expression</th>
<th>Mem B cells expression</th>
<th>active_sequence</th>
<th>chr</th>
<th>start</th>
<th>stop</th>
<th>mirbase accession</th>
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<td>6.83</td>
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<td>chr16</td>
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<td>54,385,567</td>
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<td>2.15</td>
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<td>..</td>
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<td>0.42</td>
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a Selected Test: T Test unpaired; p-value computation: Asymptotic
b Multiple Testing Correction: Benjamini-Hochberg; Corrected p-value cut-off= 0.05
c Fold change cut-off: 1.5
Table 2. Differentially expressed microRNAs between M-CLL and UM-CLL

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<th>All d</th>
<th>13q- e</th>
<th>Corrected p-value a,b</th>
<th>Fold change c</th>
<th>regulation UM/M</th>
<th>CLL [M] expression</th>
<th>CLL [UM] expression</th>
<th>active_sequence</th>
<th>chr</th>
<th>start</th>
<th>stop</th>
<th>mirbase accession</th>
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<td>1</td>
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<td>39.59</td>
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<td>chr10</td>
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<td>104,186,288</td>
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<td>3.12</td>
<td>down</td>
<td>14.02</td>
<td>4.49</td>
<td>GAAACCCAGGAAGAATCGGT</td>
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<td>207,975,215</td>
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<td>1,542.98</td>
<td>817.74</td>
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<td>3,504.23</td>
<td>CGCCAATTTAGTGTCG</td>
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<td>160,122,563</td>
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<td>hsa-miR-340</td>
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<td>3.90E-03</td>
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<td>up</td>
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<td>1.01</td>
<td>AATCAGTTCATTGCCTTA</td>
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<td>hsa-miR-338-3p</td>
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<td>1.33</td>
<td>GCTATAAGTAACGTAGAAGC</td>
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<td>1.64</td>
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<td>7.97</td>
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<td>45,606,498</td>
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<td>141,742,678</td>
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<tr>
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a Selected Test: T Test unpaired; p-value computation: Asymptotic
b Multiple Testing Correction: Benjamini-Hochberg; Corrected p-value cut-off= 0.05
c Fold change cut-off: 1.5
d 133 M-CLLs vs 82 UM-CLLs
e 70 M-CLLs vs 15 UM-CLLs
Table 3. MicroRNAs deregulated in specific FISH subgroups of CLL

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<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-value</th>
<th>active_sequence</th>
<th>chr</th>
<th>start</th>
<th>stop</th>
<th>mirbase accession No</th>
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<td>1.52</td>
<td>2.72</td>
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<td>chr19</td>
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<td>46,522,240</td>
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<td>0.98</td>
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<td>up</td>
<td>9.00E-03</td>
<td>CAACAAAATCTGTATGTG</td>
<td>chr17</td>
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<td>79,099,726</td>
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<td>hsa-miR-155</td>
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<td>12+</td>
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<td>hsa-miR-484</td>
<td>FISH neg</td>
<td>1.02</td>
<td>2.22</td>
<td>2.21</td>
<td>down</td>
<td>9.60E-05</td>
<td>ATCGGAGGGGACTGA</td>
<td>chr16</td>
<td>15,737,165</td>
<td>15,737,179</td>
<td>MIMAT0002174</td>
</tr>
</tbody>
</table>

* Selected Test: T Test unpaired; p-value computation: Asymptotic
* Multiple Testing Correction: Benjamini-Hochberg; Corrected p-value cut-off= 0.05
FIGURE 3
Clinical Cancer Research

microRNAome expression in chronic lymphocytic leukemia: comparison with normal B cell subsets and correlations with prognostic and clinical parameters

Massimo Negrini, Giovanna Cutrona, Cristian Bassi, et al.

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