Molecular Medicine

Differential microRNA Profiles and Their Functional Implications in Different Immunogenetic Subsets of Chronic Lymphocytic Leukemia

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Critical processes of B-cell physiology, including immune signaling through the B-cell receptor (BcR) and/or Toll-like receptors (TLRs), are targeted by microRNAs. With this in mind and also given the important role of BcR and TLR signaling and microRNAs in chronic lymphocytic leukemia (CLL), we investigated whether microRNAs could be implicated in shaping the behavior of CLL clones with distinct BcR and TLR molecular and functional profiles. To this end, we examined 79 CLL cases for the expression of 33 microRNAs, selected on the following criteria: (a) deregulated in CLL versus normal B-cells; (b) differentially expressed in CLL subgroups with distinct clinicobiological features; and, (c) if meeting (a) + (b), having predicted targets in the immune signaling pathways. Significant upregulation of miR-150, miR-29c, miR-143 and miR-223 and downregulation of miR-15a was found in mutated versus unmutated CLL, with miR-15a showing the highest fold difference. Comparison of two major subsets with distinct stereotyped BcRs and signaling signatures, namely subset 1 (*IGHV1/5/7-IGKV1(D)-39*, unmutated, bad prognosis) versus subset 4 (*IGHV4-34/IGKV2-30*, mutated, good prognosis) revealed differences in the expression of miR-150, miR-29b, miR-29c and miR-101, all downregulated in subset 1. We were also able to link these distinct microRNA profiles with cellular phenotypes, importantly showing that, in subset 1, miR-101 downregulation is associated with overexpression of the enhancer of zeste homolog 2 (EZH2) protein, which has been associated with clinical aggressiveness in other B-cell lymphomas. In conclusion, specific miRNAs differentially expressed among CLL subgroups with distinct BcR and/or TLR signaling may modulate the biological and clinical behavior of the CLL clones.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) was considered a homogeneous disease of naive, immune-incompetent, minimally proliferating B cells that accumulated because of intrinsic apoptotic defects (1). More recently, however, this view was essentially upturned: indeed, currently, it is widely accepted that the development and evolution of CLL reflects an interplay between genetics and microenvironment (2). Microenvironmental stimuli specifically conveyed through the B-cell receptor (BcR) are critically implicated in the immunopathogenesis of CLL as evidenced by (a) restrictions in the immunoglobulin heavy variable (*IGHV*) gene repertoire (3); (b) different prognosis of patients with different *IGHV* gene mutational status (4,5); and (c) the existence of subsets of patients sharing BcRs with restricted, quasi-identical immunoglobulin (IG) sequences (stereotyped BcRs) in roughly 30% of cases (6–8). These observations may indicate

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that a correspondingly restricted set of antigens or structurally related epitopes might be implicated in the selection of clones carrying distinctive BcRs (9).

In addition to the BcR, B cells express a wide repertoire of molecules involved in microenvironmental interactions, including Toll-like receptors (TLRs) (10). TLRs have important roles in both detecting pathogens and initiating inflammatory processes that subsequently prime specific adaptive immune responses during infection (11). Dual BcR and TLR engagement can fine-tune B-cell responses (12). However, in some instances, it may also result in aberrant activation and loss of tolerance (13), alluding to functional complementation.

Recent studies by us (14,15) and others (16) have demonstrated that CLL patient subgroups defined by specific molecular characteristics of their clonotypic BcRs have distinct patterns of TLR pathway gene expression, function and/or tolerance. These findings indicate that specific modalities of BcR/TLR collaboration and/or regulation may eventually affect the biological behavior of the malignant clones.

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by binding to specific mRNA targets and promoting their degradation and/or translational inhibition (17). miRNAs participate in the regulation of diverse cellular processes, from development, differentiation and cell cycle regulation to senescence and metabolism (18) and the modulation of immune signaling (19,20). Moreover, an intertwined connection between epigenetics and miRNAs has been supported by the identification of a specific subgroup of miRNAs called "epi-miRNAs" that can modulate the activity of the epigenetic machinery. The complexity of this connection is enhanced by the epigenetic regulation of miRNA expression that generates a fine regulatory feedback loop (21).

Taking all the above into consideration, it is no surprise that miRNAs have been found to be instrumental for CLL physiology (22). In particular, certain miRNAs are deregulated in CLL compared with normal B cells (23–26), whereas distinct microRNA signatures are associated with prognostic factors and disease progression (24–29), response to treatment (30,31) and various other disease characteristics (26,32,33).

Prompted by these findings, we investigated whether miRNAs could be implicated in shaping the biological and, by logical inference, clinical behavior of subsets of patients with CLL with particular BcR molecular features and distinct BcR and/or TLR molecular and functional profiles. We report that specific miRNAs differentially expressed among different CLL subsets can affect their cellular physiology, at least under certain *ex vivo* conditions. We also provide for the first time molecular and functional evidence linking overexpression of the enhancer of zeste homolog 2 (EZH2) protein in clinically aggressive CLL with downregulation of miR-101. Altogether, these findings argue for the operation of subset-specific miRNA-regulated processes that may eventually affect the evolution of CLL clones with distinct BcRs and immune signaling signatures.

Table 1. Clinical and biological data of the patient cohort.

Parameter	*n*
Sex (M/F)	52/27
Binet stage at diagnosis (A/B/C)	67/9/3
CD38 expression (positive/negative)	28/50
IGHV gene mutational status (mutated/unmutated)	45/34

MATERIALS AND METHODS

Patient Group

Blood samples were collected from 79 patients diagnosed with CLL according to the recently revised guidelines of the International Workshop Chronic Lymphocytic Leukemia/National Cancer Institute (34). All patients were either untreated or off therapy for at least 6 months before the study. The study was approved by the local ethics review committee of the participating institutions. Demographic, clinical and biological data for the patient cohort are given in Table 1 and Supplementary Table S1.

Polymerase Chain Reaction Amplification and Sequence Analysis of IGHV-IGHD-IGHJ Rearrangements

Reverse transcriptase–polymerase chain reaction (RT-PCR) amplification and sequence analysis of IGHV-IGHD-IGHJ gene rearrangements was performed as previously described (8).

Cell Purification

$CD19^+$ B cells were negatively selected from whole blood using the RosetteSep B-cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) following the manufacturer's instructions. The purity of all cell preparations always exceeded 95% for $CD19^+$ cells.

miRNA Isolation and Reverse Transcription of Mature miRNAs

Small RNA fractions (<200 nucleotides) were extracted with the TRIsure Reagent (Bioline, London, UK) and the RT^2 qPCR-Grade miRNA Isolation Kit (SABiosciences, Valencia, CA, USA). Then 100 ng purified RNA was used for the synthesis of cDNA with the RT^2 miRNA First Strand Kit (SABiosciences).

Identification of miRNAs Potentially Implicated in the Regulation of Immune Signaling Pathways in CLL

On the basis of published work, we identified a series of miRNAs reported as relevant for CLL biology: (a) miRNAs deregulated in CLL cells compared with normal B cells (24,25,35) and (b) miRNAs differentially expressed in subgroups of CLL patients with distinct profiles of biological prognostic markers (24,25,28,33) (Supplementary Table S2). Through the use of our purpose-built computational

algorithms and tools (DIANA-microT v3.0 algorithm and DIANA-mirPath tool v1.0; both freely available at http://microrna.gr), we investigated which of these miRNAs are potentially implicated in the regulation of biological processes relevant for B cells, with a focus on immune signaling. In particular, we evaluated the BcR and TLR pathways as well as the mitogen-activated protein kinase (MAPK) pathway, which integrates with both of the above. We selected for bioinformatics analysis those molecules in the aforementioned pathways for which gene expression data were available from our previous work (14). Through this approach, we identified 33 miRNAs with predicted targets in the examined pathways (Supplementary Tables S3 and S4). Details about the bioinformatics analytical strategy are given in the Supplementary Methods.

Quantification of miRNA Expression

Expression profiling of all 33 mature miRNAs listed in Supplementary Table S3 was performed by real-time quantitative PCR (RQ-PCR) on customized PCR arrays (SABiosciences), consisting of a panel of 41 primer sets used for the amplification of the target microRNAs plus four reference small noncoding RNAs (snRNA U6, SNORD44, SNORD47 and SNORD48), two reverse transcription controls and two PCR quality controls. Further details are provided in the Supplementary Methods.

Quantification of EZH2 mRNA Expression

Total RNA was extracted from cells with the use of TRIsure (Bioline, London, UK). Quantification of EZH2 mRNA levels was achieved by RQ-PCR, by using the RT^2 qPCR Primer Assay for the human *EZH2* gene (SABiosciences), according to the manufacturer's instructions and as detailed in the Supplementary Methods.

Western Blot Analysis

Total cellular protein was isolated from purified B cells, run on 10% NuPAGE Bis-Tris gel (Invitrogen/Life Technologies, Carlsbad, CA, USA) and transferred to polyvinylidene fluoride membranes (Invitrogen/Life Technologies) as reported previously (15). Immunoblot analysis was performed for certain proteins identified through bioinformatics analysis as potential targets of differentially expressed miRNAs (namely EZH2, mitogen-activated protein kinase 8 [MAPK8] and cellular FBJ murine osteosarcoma viral oncogene homolog [c-FOS]) by using appropriate mouse monoclonal anti-human antibodies (EZH2; BD Biosciences, San Jose, CA, USA; MAPK8 and FOS, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Further details are given in the Supplementary Methods.

Nucleofection of CLL Cells

Purified CD19$^+$ B cells from one subset 1 and one subset 4 case were transfected with specific miRNA mimic and miRNA inhibitor, respectively, by using the Amaxa Human B Cell Nucleofector Kit (Lonza, Colonge, Germany). In detail, 5×10^6 CD19$^+$ B cells of each case were resuspended in 100 μL Human B Cell Nucleofector Solution and mixed with 40 nmol/L Syn-hsa-miR-101-3p miScript miRNA Mimic (Qiagen, Hilden, Germany) in the case of subset 1 and 100 nmol/L Anti-hsa-miR-101-3p miScript miRNA Inhibitor (Qiagen) in the case of subset 4.

The 5×10^6 CD19$^+$ B cells of each case were also transfected with the appropriate negative control. In the subset 1 case, 40 nmol/L AllStars Negative Control siRNA (Qiagen) was used, whereas 100 nmol/L miScript Inhibitor Negative Control (Qiagen) was used in the subset 4 case. The same amount of cells was cultured as untranfected control. Nucleofections were performed by using the Amaxa Nucleofector device with the U-15 program (Lonza). Transfection efficiency was measured with the Block-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen/Life Technologies). CLL cells were collected and processed for immunoblotting analysis at 24 and 40 h after nucleofection.

Statistical Analysis

Descriptive statistics were performed as previously described (14,15). Correlation analysis was performed by using the Pearson correlation coefficient. For all comparisons, a significance level of $p \leq 0.05$ was set. All statistical analyses were performed with the use of the GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

All supplementary materials are available online at www.molmed.org.

RESULTS

IGHV Repertoire and Mutational Status

IGHV-IGHD-IGHJ sequences were available for all studied cases (Supplementary Table S5). Following the 98% germline identity cutoff value (4,5), 45 of 79 sequences (57%) were defined as mutated, whereas the remainder (34 of 79 sequences, 43%) had unmutated *IGHV* genes; in the latter group, 25 cases carried *IGHV* genes with 100% identity to the germline.

The study was intentionally biased to cases expressing stereotyped BcRs assigned to subset 1 [stereotyped *IGHV1/5/7-IGKV1(D)-39* BcRs, seven cases] and subset 4 [stereotyped *IGHV4-34/IGKV2-30* BcRs, eight cases] (Supplementary Table S5) (8). These subsets may be seen as prototypes of "unmutated/adverse prognosis" and "mutated/good prognosis" subsets, respectively. Furthermore (perhaps more importantly for the purposes of the present study), these subsets also exhibit distinct immune signaling profiles (14,15). All subset 1 cases carried unmutated *IGHV* genes, whereas, in contrast, all subset 4 cases carried mutated *IGHV* genes.

miRNA Expression Profiling: Analysis at the Cohort Level

A total of 33 miRNAs predicted to target the BcR and/or TLR pathways were evaluated. Significant differences were identified with regards to miRNA expression levels, which ranged from high

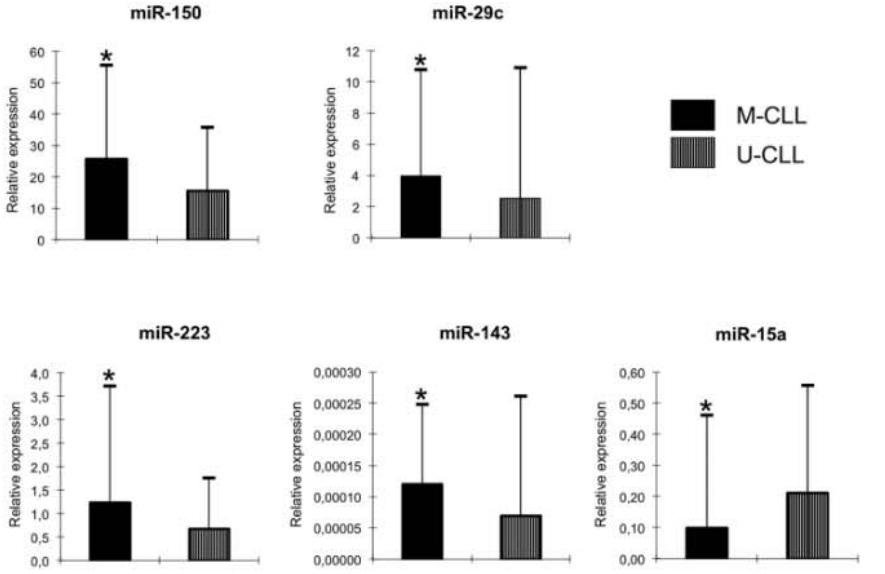


Figure 1. Differential miRNA expression in M-CLL versus UM-CLL. Only miRNAs with fold difference in expression of ≥1.50 and significantly different expression in M-CLL versus U-CLL ($p \leq 0.05$) are represented in the graphs. Among them, miR-15a, downregulated in M-CLL, has the highest fold difference. The *y* axis depicts the relative expression ($2^{-\Delta Ct}$). Error bars represent standard deviation (SD). *$p \leq 0.05$ compared with U-CLL.

to undetectable (Supplementary Table S6; Supplementary Figure S1). Additionally, for most studied miRNAs, significant interpatient variation was observed. Statistical analysis of miRNA expression profiles with various clinical parameters, including stage at diagnosis and time to first treatment, did not identify significant associations, likely because of (a) the relatively small size of the cohort and (b) case selection.

miRNA Expression Profiles in Relation to BcR Molecular Features

We sought for differences in miRNA expression in subgroups of cases defined by BcR molecular features, such as the mutational status of the clonotypic *IGHV* genes and the expression of stereotyped BcRs.

The comparison between CLL cases carrying mutated versus unmutated *IGHV* genes (mutated [M]-CLL and unmutated [U]-CLL: 45 and 34 cases, respectively) revealed differential expression of nine miRNAs (Supplementary Table S7A). Amongst these, the most pronounced differences ($p < 0.05$) were seen for miR-150, miR-15a, miR-29c, miR-223 and miR-143. The greatest difference overall was recorded for miR-15a, which was the only downregulated miRNA in M-CLL (Figure 1).

We explored whether the observed differences regarding miR-15a expression between M-CLL versus U-CLL could relate to the genomic status at chromosome 13q14, which harbors the miR-15a locus (22). Among 59 cases with available interphase fluorescence *in situ* hybridization (FISH) data, 7 (11.9%) and 19 (32.2%) carried biallelic or monoallelic del(13q), respectively, whereas the remainder (33 cases, 55.9%) did not exhibit aberrations of chromosome 13q. The majority of cases carrying del(13q) [5 of 7 cases with biallelic del(13q) and 13 of 19 cases with monoallelic del(13q)] carried mutated *IGHV* genes.

Cases with biallelic del(13q) exhibited significantly downregulated miR-15a levels compared with cases with either monoallelic del(13q) ($p = 0.002$) or no detectable chromosome 13q aberration ($p < 0.001$) (Supplementary Figure S2). Monoallelic del(13q) cases also exhibited downregulated miR-15a levels compared with cases with no detectable chromosome 13q aberration, although not reaching statistical significance ($p = 0.07$).

We next evaluated differences in miRNA expression between clinically aggressive stereotyped subset 1 (U-CLL) versus clinically indolent stereotyped subset 4 (M-CLL). We found 12 differentially expressed miRNAs, all downregulated in subset 1, with miR-150, miR-29b, miR-29c and miR-101 showing statistically significant difference between the two subsets ($p \leq 0.05$) (Figure 2, Supplementary Table S7B). However, miR-150 and miR-29c appeared as differentially expressed between M-CLL versus U-CLL, whereas miR-29b and miR-101 showed subset-biased differential expression irrespectively of mutational status.

Impact of Differentially Expressed miRNAs on Immune Signaling in CLL

Given that CLL cases with distinct immunogenetic features also exhibit distinct responses to immune triggering (15,16,36), we next investigated if and how the differentially expressed miRNAs might be implicated in regulating the function of immune signaling pathways. We focused on miRNAs, showing the highest fold difference between the groups under comparison, namely miR-15a for M-CLL versus U-CLL and miR-101 for subset 1 versus 4. Among all predicted targets of these two miRNAs in the examined pathways, we selected for analysis those for which gene expression data was available from our recent profiling study of immune signaling in CLL (14).

In the case of miR-15a, potential targets identified through bioinformatics analysis included MAPK8, IRAK2, CD80 and IKBKB, with MAPK8 having the highest prediction score (Supplementary Table S8A). A significant negative correlation ($r = -0.3$, $p < 0.01$) was identified between miR-15a and MAPK8 mRNA levels (Figure 3A). In contrast, no correlation existed between miR-15a and IRAK2, CD80 or IKBKB mRNA levels. Contrary to expectations, comparison of MAPK8 protein expression in seven M-CLL ver-

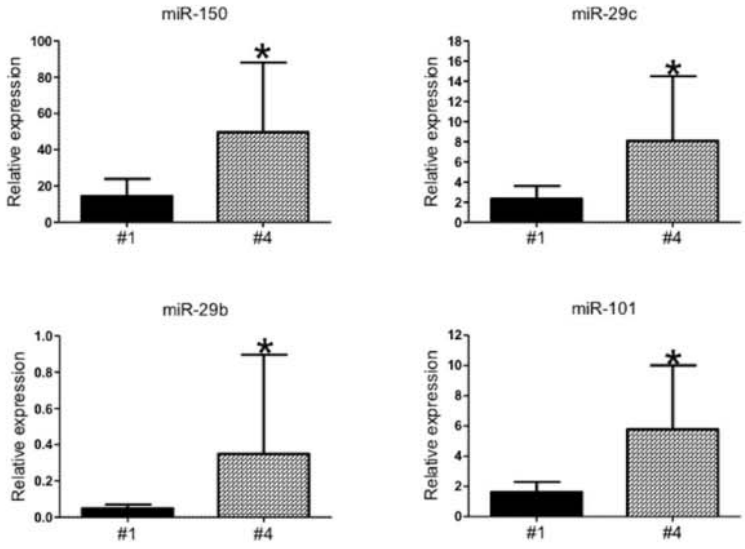


Figure 2. Differentially expressed miRNAs in subset 1 versus 4 CLL cases. Only miRNAs with fold difference in expression of ≥1.50 and significantly different expression ($p \leq 0.05$) are represented in the graphs. Among them, miR-101, downregulated in subset 1, shows the highest fold difference. The *y* axis depicts the relative expression ($2^{-\Delta Ct}$). Error bars represent SD. *$p \leq 0.05$ compared with subset 4.

sus seven U-CLL cases revealed significantly ($p < 0.05$) increased levels in U-CLL (Figure 3B). Given that U-CLL also show elevated levels of miR-15a, this result indicates additional modes of regulation of MAPK8 expression.

In the case of miR-101, FOS and MAPK8 were identified as potential targets, with FOS having the highest prediction score (Supplementary Table S8B) and being reported to be regulated by miR-101 in other types of cancer (37,38). We found a negative correlation ($r = -0.8$, $p < 0.01$) between miR-101 and FOS mRNA levels (Figure 3C), but not with MAPK8 mRNA levels. Western blot analysis revealed similar FOS protein levels between subset 1 and 4 cases (Figure 3D).

Next we investigated the impact of miR-101 forced overexpression in primary CLL cells. We transfected CLL cells from one subset 1 case with miR-101 mimic, and, after 40 h in culture, we observed downregulation of FOS protein levels compared with untransfected ells or cells transfected with negative control (Figure 3E). Furthermore, we found that transfecting subset 4 cells with miR-101 inhibitor induced FOS downregulation, yet to a lesser extent compared with miR-101 forced overexpression (Figure 3F). These results indicate that miR-101 may be implicated in the regulation of FOS expression in CLL.

miR-101 Modulates the Expression of the EZH2 Methyltransferase in Different CLL Subsets

miR-101 is considered an "epi-miRNA," since it targets EZH2, a histone methyltransferase that mediates transcriptional repression acting in concert with DNA methyltransferases (21). In both solid tumors and hematopoietic malignancies, more aggressive subtypes were associated with overexpression of EZH2 caused by different mechanisms, including genomic loss of the miR-101 locus (39–41). Relevant to the latter observation, induced expression of miR-101 in cancer cell lines inhibits the expression and function of EZH2 (42).

With this in mind, and also prompted by our finding of significant downregulation of miR-101 in subsets 1 versus 4, we investigated the potential role of miR-101 in regulating EZH2 expression in these paradigmatic CLL subsets. We detected significantly higher levels of EZH2 mRNA in subset 1 ($p < 0.05$) (Figure 4A) that negatively correlated ($r = -0.75$, $p < 0.05$) with miR-101 expression (Figure 4B). We also found significantly higher EZH2 protein expression in subset 1 versus subset 4 ($p < 0.05$) (Figure 4C), again showing significant negative correlation ($r = -0.7$, $p < 0.05$) with miR-101 expression levels (Figure 4D).

Finally, we investigated the impact of (a) forced overexpression and (b) downregulation of miR-101 in primary CLL cells. In subset 1 CLL cells, the miR-101 mimic induced downregulation of EZH2 protein levels after 40 h in culture compared with untransfected cells or cells transfected with negative control (Figure 4E). In subset 4 CLL cells transfected with miR-101 inhibitor, upregulation of EZH2 protein levels was seen after 24 h compared with untransfected cells or cells transfected with negative control (Figure 4F). Hence, not only is miR-101 differentially expressed in different CLL subsets, but also (at least partly) regulates EZH2 expression, potentially leading to differential biological effects.

DISCUSSION

Immune-mediated pathways are important in the pathogenesis of CLL (14–16,36,43). Indeed, the *in vivo* accumulation of CLL clonal cells is promoted by classic receptor-ligand interactions with other cells and soluble factors occurring within the tumor microenvironment (2). However, CLL cells from different patients exhibit heterogeneous functional responses when stimulated through immune receptors, that is, the BcR and/or the TLRs (14–16,36,43), prompting the investigation into the underlying mechanisms.

Recent evidence suggests that miRNAs target key molecules of the B-cell immune pathways (20,44,45). With this in mind and also taking into account that several miRNAs are differentially expressed in CLL, we investigated whether miRNAs could also be implicated in the

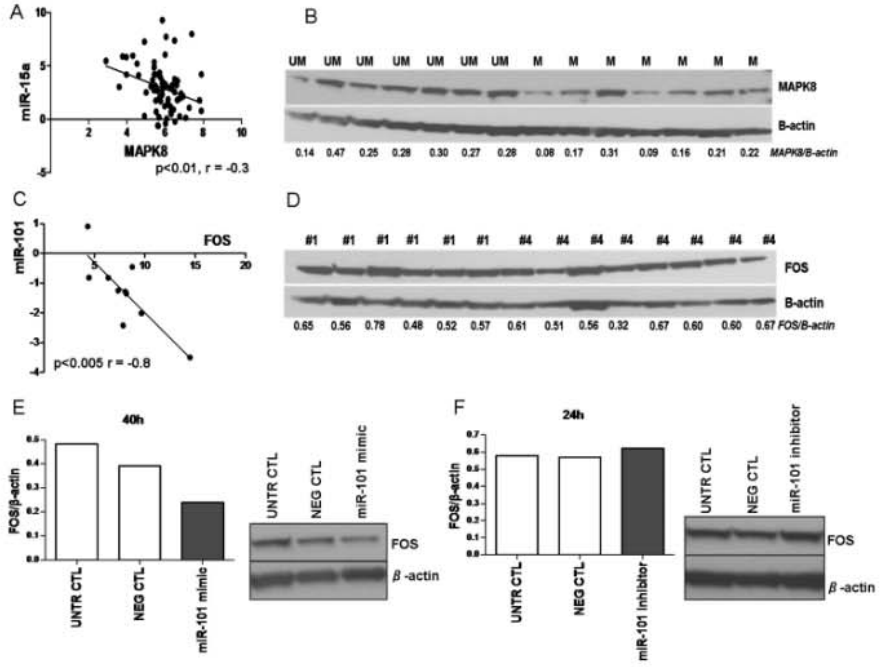


Figure 3. miR-15a/MAPK8 and miR-101/FOS are negatively correlated at mRNA but not at protein level. MAPK8 (JNK) is the target with the highest prediction score for miR-15a. (A, C) Correlation and linear regression between miR-15a/MAPK8 and miR-101/FOS mRNA levels, respectively. ΔCt values were used and the correlation is statistically significant ($p < 0.01$). The slope in both cases is negative in both cases, which means that when miRNA expression is upregulated, mRNA expression is downregulated. (B, D) Protein levels of MAPK8 and FOS in representative M/U-CLL and subset 1 and 4 cases, respectively. MAPK8 is overexpressed in U-CLL. (E) miR-101 forced overexpression in one subset 1 case by using miR-101 mimic induced FOS protein downregulation compared with untransfected cells or cells transfected with negative control. (F) Transfection of subset 4 cells with miR-101 inhibitor induced FOS downregulation, yet to a lesser extent compared with miR-101–forced overexpression.

distinct immune signaling profiles of CLL subgroups with different clinical presentation and outcome. In particular, we profiled a series of well-characterized CLL patients for the expression of 33 miRNAs verified as relevant for CLL biology and also identified as potential modulators of immune signaling pathways in B cells.

Comparison of miRNA expression between M-CLL and U-CLL cases of the present study confirms and extends previous reports. In particular, our finding of overexpression of miR-150, miR-29c, miR-223 in M-CLL and miR-15a in U-CLL is in accordance with the literature (24,25,28), whereas the relative upregulation of miR-143 in M-CLL versus U-CLL is reported for the first time. Among the differentially expressed miRNAs in M-CLL versus U-CLL, miR-15a showed the highest fold difference; miR-15a levels were also strongly associated with deletion of chromosome 13q14, in line with previous observations (22,46).

CLL subgroups defined by *IGHV* gene mutational status are not homogeneous. Rather, within each mutational subgroup, cases assigned to subsets expressing distinct stereotyped BcR have been shown to share distinctive clinicobiological profiles and outcome (47). On this basis, we narrowed down our comparisons to cases assigned to different subsets with stereotyped BcR with a special focus on subsets 1 and 4, which were overrepresented in our cohort for both practical and scientific reasons.

In particular, because even the most populated stereotyped subsets account for only a minor fraction of the cases with available IGHV-IGHD-IGHJ sequence information, the choice to study subsets 1 and 4, the most populated subsets among U-CLL and M-CLL, respectively (6,8,47), offered us the possibility to obtain a sizeable collection of samples for meaningful experimental analysis and statistical evaluation. Admittedly, numbers are still relatively low, as in all previous studies analyzing stereotyped subsets compared with studying CLL in general (47). That being said, the subsets selected for analysis are opposites in terms of clinical evolution and outcome (subset 1: aggressive disease; subset 4: remarkably indolent disease) (8,47). Furthermore, they exhibit consistent yet distinct immune signaling profiles, even when compared with non-subset cases of similar *IGHV* gene mutational status (14,15). Thus, the subsets can be considered as prototypes of distinct biological and clinical setting.

Comparison of these two subsets revealed 12 differentially expressed miRNAs, all downregulated in subset 1, with miR-101 showing the most pronounced difference in expression between subsets 1 (low) versus 4 (high). Interestingly, no differences in miR-101 expression were identified when we compared M-CLL versus U-CLL; hence, the differential profiles in subsets 1 and 4 are not a mere reflection of differential *IGHV* gene mutational status; rather, they imply a role for miR-101 in shaping the biological behavior of the respective clones.

miR-101 is one of the so-called epi-miRNAs, a group of miRNAs that regulate the expression of components of the epigenetic machinery. Aberrant expression of epi-miRNAs has been linked to the development or progression of human cancer (21). miR-101 regulates EZH2, the enzymatic subunit of the polycomb repressive complex 2 (PRC2), which induces gene repression through trimethylation of histone H3 at lysine 27. EZH2 overexpression caused by different mechanisms, includ-

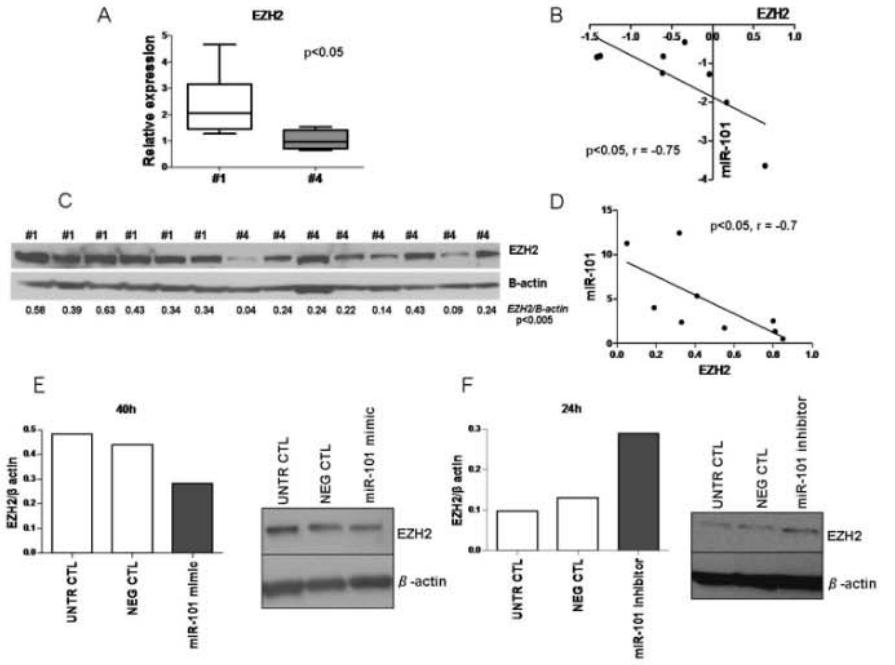


Figure 4. miR-101 differentially regulates EZH2 at mRNA and protein levels in subset 1 versus 4 CLL cases. (A) EZH2 mRNA levels in subsets 1 and 4 (four and four cases, respectively). The *y* axis represents relative expression ($2^{-\Delta Ct}$). The box-and-whisker plots repesent median with minimum and maximum values. EZH2 is upregulated in subset 1 cases (fold difference in expression of 2.3; $p \leq 0.05$). (B) Correlation and linear regression between miR-101 and EZH2 mRNA levels. ΔCt values were used and the correlation is statistically significant ($p \leq 0.05$). The slope is negative, which means that when miR-101 expression is upregulated, EZH2 expression is downregulated ($r = -0.75$). (C) Protein levels of EZH2 in subsets 1 and 4 (six and eight cases, respectively). EZH2 protein levels are higher in subset 1 ($p < 0.005$). (D) Correlation and linear regression between miR-101 relative expression ($2^{-\Delta Ct}$) and EZH2 protein levels ($r = -0.7$, $p < 0.05$). (E) In subset 1 CLL cells, miR-101 mimic induces downregulation of EZH2 protein levels compared with untransfected cells and cells transfected with negative control. (F) In subset 4 CLL cells, miR-101 inhibitor induces EZH2 protein upregulation compared with untransfected cells or cells transfected with negative control.

ing activating mutations, was reported in a broad range of both hematopoietic and solid human malignancies and associated with poor prognosis (48,49).

Here, we provide for the first time evidence that EZH2 might be implicated in shaping the distinct behavior of CLL subsets 1 versus 4 and that its expression is regulated, at least in part, by miR-101. In particular, we report a significant inverse correlation between EZH2 mRNA and protein with miR-101 expression, suggesting that increased EZH2 expression in clinically aggressive subset 1 is linked to downregulation of miR-101, alluding to the operation of a regulatory loop. This scenario is also supported by our finding that induced overexpression or downregulation of miR-101 affected EZH2 protein expression in primary CLL cells from subsets 1 and 4.

With this evidence that miRNAs with distinct expression profiles among different CLL subgroups with distinct BcR IGs can be linked to the behavior of the respective CLL cases, we next sought to investigate the possible impact of differentially expressed miRNAs on the expression of key molecules of immune signaling pathways.

In particular, we demonstrate for the first time a significant inverse correlation of miR-15a with MAPK8 mRNA expression in M-CLL versus U-CLL, albeit with no correlation between miRNA levels and protein levels. A similar situation was identified for miR-101 and FOS expression in subsets 1 versus 4 (namely association between miRNA and mRNA yet not with protein levels). These observations are in line with previous reports that miR-101 regulates FOS mRNA expression in gastric cancer (38), whereas the available data about protein regulation by miR-101 overexpression in cancer cell lines (40) and human hepatocellular carcinoma (37) are inconclusive. Interestingly, we found that forced overexpression of miR-101 downregulated FOS protein levels in primary CLL cells.

miRNAs cause inhibition of translation or degradation of the target mRNA. However, as recently reported, they predominantly affect mRNA rather than protein levels (50). Of note, the degree of the protein downregulation imposed by miRNAs often tends to be quantitatively modest (even an overexpressed miRNA typically downregulates most of its endogenous targets by <50%) (51). Furthermore, as already suggested, miRNAs might fully control the expression of immune signaling proteins through collaboration with multiple other mechanisms, including physical interactions, conformational changes, posttranslational modifications and proteasome-mediated degradation. These findings may be relevant for the observed inconsistencies between miRNA, mRNA and protein expression of MAPK8 and FOS in the studied CLL cases, indicating the need for further investigation. All that notwithstanding, it is worth mentioning that our analyses were conducted at basal level without any form of stimulation, which is relevant in view of recent reports by us (52) and others (44) that the triggering of immune receptors modulates miRNA expression in CLL, alluding to autoregulatory control.

CONCLUSION

We demonstrate that subgroups of CLL cases with distinct BcR IGs are character-

ized by distinct miRNAs profiles that can potentially be implicated in the regulation of immune signaling (49). Furthermore, we provide for the first time evidence that miR-101 regulates EZH2 expression levels in CLL and that EZH2 overexpression is a feature of aggressive CLL.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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