**MicroRNA-155 Influences B-cell Receptor Signaling And Associates With Aggressive Disease In Chronic Lymphocytic Leukemia**

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Short title:  *miR-155* regulates BCR-signaling in CLL

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Key Points

High-level miR-155 enhances B-cell-receptor (BCR) signaling, and is associated with poor prognosis in CLL.

Signals within the CLL microenvironment, such as CD154 or BAFF, can induce miR-155 and enhance BCR signaling.
Abstract

High-level leukemia-cell expression of miR-155 is associated with more aggressive disease in patients with chronic lymphocytic leukemia (CLL), including those cases with low-level expression of zeta-chain associated protein of 70 kD (ZAP-70). CLL with high-level miR-155 expressed lower levels of Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) and were more responsive to B-cell-receptor (BCR) ligation than CLL with low-level miR-155. Transfection with miR-155 enhanced responsiveness to BCR ligation, whereas transfection with a miR-155 inhibitor had the opposite effects. CLL in lymphoid tissue expressed higher levels of miR155HG than CLL in the blood of the same patient. Also, isolated CD5brightCXCR4dim cells, representing CLL that had been newly-released from the microenvironment, expressed higher levels of miR-155 and were more responsive to BCR ligation, than isolated CD5dimCXCR4bright cells of the same patient. Treatment of CLL or normal B-cells with CD40-ligand or B-cell-activating factor (BAFF) upregulated miR-155, and enhanced sensitivity to BCR ligation, effects that could be blocked by inhibitors to miR-155. This study demonstrates that the sensitivity to BCR ligation can be enhanced by high-level expression of miR-155, which in turn can be induced by cross-talk within the tissue microenvironment, potentially contributing to its association with adverse clinical outcome in patients with CLL.

Keywords: CLL, microenvironment, miR-155, BCR signaling, survival
**Introduction**

Signaling via the B-cell receptor (BCR) is thought to play a role in the pathogenesis and/or progression of chronic lymphocytic leukemia (CLL).\(^1,^2\) BCR signaling is triggered by phosphorylation of immunoreceptor tyrosine-based activation motifs of CD79a/b and induces a cascade of downstream signaling pathways.\(^3,^4\) The importance of this cascade in CLL biology appears underscored by clinical trials demonstrating clinical activity of small-molecule kinase inhibitors that can block BCR-signaling.\(^5\)

The biology of CLL also can be influenced by microRNAs.\(^6-^10\) In particular, the evolutionarily-conserved microRNA *miR-155* is a critical regulator of post-transcriptional gene expression in B-cells.\(^6\) It is encoded within a region known as the B-cell integration cluster (*BIC, miR155HG*), which originally was identified as being a frequent integration site for avian leucosis virus.\(^11-^13\) This microRNA is over-expressed in numerous B-cell lymphomas,\(^14-^16\) including chronic lymphocytic leukemia (CLL),\(^8,^{17-20}\) suggesting that *miR-155* contributes to lymphoma development.\(^19\) Consistent with this notion is the observation that mice made transgenic for *miR-155* under a B-cell-specific promoter developed pre-B-cell lymphomas.\(^21\) Specifically high levels of *miR-155* also are present in diffuse large B-cell lymphomas with an activated-B-cell phenotype, which is associated with a relatively poor clinical prognosis.\(^14\) Moreover, relatively high-level expression of *miR-155* in CLL has been associated with expression of adverse prognostic markers, such as the zeta-chain associated protein of 70 kD (ZAP-70), unmutated IGHV, and/or deletions in 17p or 11q.\(^22-^25\) Over-expression of *miR-155* in transgenic mice induces polyclonal B-cell expansion, suggesting that *miR-155* could enhance B-cell proliferation.\(^21\)

One identified target of *miR-155* is the Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1), which is encoded by *INPP5D*.\(^26,^27\) SHIP1 is phosphatase that acts
in opposition to kinases, which are integral to many signal transduction pathways. Such inhibitory phosphatases may suppress surface immunoglobulin and B-cell receptor (BCR) signaling. Such signaling appears most prominent in lymph nodes (LN), where CLL cells apparently become activated in response to signals from the LN microenvironment. Moreover, CLL cells in the LN have up-regulated BCR gene-expression signatures and higher proportions of cells undergoing proliferation than CLL cells in the blood. Conceivably, the expression of miR-155 could influence the relative expression of SHIP1 in CLL, which then could influence the relative activation of signaling pathways triggered by ligation of the BCR by self- or environmental- antigen(s). We hypothesize that high-level expression of miR-155 in CLL can repress expression of SHIP1 and increase the responsiveness to BCR ligation, thus possibly accounting for its association with adverse clinical outcome in patients with CLL.
Materials and Methods

Cells And Sample Preparation

Blood samples were collected from consenting patients who satisfied diagnostic criteria for CLL and enrolled in UC San Diego Moores Cancer Center biorepository, as per a protocol approved by the institutional review board (IRB) (080918). At the time of sample collection, patients had not received prior therapy. IGHV mutation status and ZAP-70 expression were assessed as per established criteria.\textsuperscript{31} We used Ficoll-Hypaque density-gradient centrifugation to obtain mononuclear cells of which $\geq 95\%$ were CD5$^+$CD19$^+$ cells. Descriptions of cell-cell transfection, measurement of intracellular calcium flux, real-time PCR, flow cytometry, and statistical analyses are provided in the supplemental methods. This study was conducted in accordance with the Declaration of Helsinki.
Results

High-level Expression Of miR-155 Is Associated With Adverse Clinical Outcome

We studied the relationship between the relative leukemia-cell expression of miR-155 and treatment-free survival (TFS), or overall survival (OS), in a cohort of eighty-six CLL patients (Table 1 and S1), for which we assayed miR-155 expression levels using absolute real-time PCR. Forty-three of the samples used mutated IGHV and the other 43 used unmutated IGHV; 41 of the samples lacked expression of ZAP-70, whereas 45 expressed ZAP-70. We used the profile-likelihood method in a Cox regression model of TFS to determine the optimal threshold level of miR-155 that might segregate these patients into 2 subgroups with disparate progression tendencies. Thirty-one patients were stratified into a “miR-155-Low” subgroup and 55 patients were assigned to a “miR-155-High” subgroup, defining a cutoff point of 2,553 copies per CLL cell. The subgroup of patients in the miR-155-High subgroup had a median TFS of 4.4 years, whereas those patients in the miR-155-Low subgroup had a median TFS of 11.4 years (Figure 1A). The median OS for patients in the miR-155-High subgroup (11.3 years) also was shorter than that of patients in the miR-155-Low subgroup (>20 years) (Figure S1). Triplicate analyses using relative real-time PCR (miR-155 versus RUN6B) demonstrated that the CLL cells in the miR-155-High subgroup had 2.4-fold higher mean level of miR-155 than did the CLL cells in the miR-155-Low subgroup (6.6 ± 0.5 verses 2.8 ± 0.3, Figure S2).

Although there was a significant association between expression of high-level miR-155 and ZAP-70 or use of unmutated IGHV (Figure S3), these associations were not absolute. Some of the CLL samples in the miR-155-High subgroup did not express ZAP-70 (n=24) or used mutated IGHV (n=17); such cases had a mean level of miR-155 that did not differ significantly from that of cases in the miR-155-High subgroup that were ZAP-70 positive or used unmutated IGHV. Similarly, some of the CLL samples in the miR-155-Low subgroup expressed ZAP-70
We used an independent data set of 181 additional patients, who had expression-levels of the \textit{miR-155} precursor measured by microarray (GSE13159, GSE13164).\textsuperscript{32} The median follow-up time for this validation cohort was 7.6 years with 64.1\% (n=116) of patients receiving therapy and 24.3\% patients deceased (n=44), similar to the original cohort. In the original collection of 86 samples, the mature \textit{miR-155} measured by qPCR was well correlated its precursor measured by microarray \((r=0.6, \ p<0.001, \text{Figure S4})\). We thus calibrated the cut-point established from the qPCR assay to the microarray assay using linear regression on these original 86 samples (Figure S4). We then validated the association of high-level expression of \textit{miR155} (as measured by the microarray) with reduced TFS and OS in the 181 new subjects, using the optimal cut-point established through analysis on the original 86 subjects. From the analysis of these new, independent data, we observed that high-level expression of \textit{miR-155} retained its strong predictive effect for TFS (Figure 1D), both when considered alone (HR 1.7; 95\% CI 1.1, 2.4) or in a model that contained both IGVH mutational status and ZAP-70 status (Table 2). In addition, greater OS was observed for the \textit{miR-155}-High group, although this did not attain statistical significance in univariate analysis (Figure S1B). In multivariate Cox models, we confirmed the independent prognostic information of \textit{miR-155} over and above the established risk factors IGVH and ZAP-70 (Table 2), both for TFS (HR 1.52; 95\% CI 1.03 2.23) and for OS (HR 2.22; 95\% CI 1.08, 4.54), using the validation cohort. Moreover, among patients with CLL cells that lacked expression of ZAP-70, those with high–levels of \textit{miR-155} (n=56) had a shorter observed median TFS (6.0 years) than patients with low-level expression of \textit{miR-155} (n = 54, 9.6 years, Figure 1E). Among patients with CLL cells that expressed mutated IGVH genes, high–levels of \textit{miR-155} (n=48) were associated with significantly shorter median TFS (6.7 years) than those with low-level
expression of miR-155 (n= 52, 18.3 years, Figure 1F).

**Relationship Between High-level Expression Of miR-155 And Expression Of SHIP1 In CLL**

We examined CLL cells for expression of SHIP1 by flow cytometry. In other cell types miR-155 has been noted to target the gene encoding SHIP1, namely INPP5D. However, miR-155 had not been reported to influence expression of SHIP1 in CLL cells. We found that CLL cells in the “High miR-155” subgroup (n=21) had a median mean fluorescence intensity ratio (MFIR) when stained for SHIP1 of 6.4 (± 0.3), which was significantly lower than that for CLL cells in the “Low miR-155” subgroup (8.0 ± 0.4, n=24, p<0.01) (Figure 2A, Figure S5A).

To further evaluate the relationship between expression of SHIP1 and miR-155 in primary CLL cells, we transfected CLL cells with a mimetic of miR-155. Transfection of CLL cells with the miR-155 mimetic reduced expression of SHIP1, as assessed by immunoblot or flow cytometric analysis (n=5, Figure 2B top, Figure S5B-C). Moreover, the median MFIR of samples stained for SHIP1 was significantly lower in cells transfected with the miR-155 mimetic than in control-transfected cells (4.4 ± 1.3 versus 6.5 ± 1.2, n=3, p <0.05) (Figure 2B bottom). Conversely, we transfected CLL cells with inhibitors of miR-155. CLL cells transfected with a miR-155 inhibitor were found to express higher levels of SHIP1, as assessed by immunoblot analysis or flow cytometry (5.7 ± 0.8 versus 4.0 ± 0.9, n=3, p <0.05) (Figure 2C). Collectively, these studies demonstrate that expression of SHIP1 in primary CLL cells is influenced by the relative expression of miR-155.

**Relationship Between Relative Expression Of miR-155 And Sensitivity to BCR Ligation**

CLL cells were stimulated with anti-µ, which rapidly induced a calcium flux that could be assessed via flow cytometry. The median increase in fluorescence intensity of calcium flux
induced by BCR ligation inversely correlated with SHIP1 expression in CLL cells (Figure S6). The median increase in calcium flux induced by anti-µ was significantly greater for CLL cells in the miR-155-High subgroup (3.4 ± 0.6) than in CLL cells in the miR-155-Low subgroup (1.2 ± 0.3, n=10, p<0.01) (Figure 3A). Transfection of the cells with a miR-155 mimetic enhanced the magnitude of calcium flux induced by treatment with anti-µ (Figure 3B). The median increase in fluorescence intensity of calcium induced by treatment of CLL cells with anti-µ was significantly greater in miR-155-transfected cells than in control-transfected CLL cells (Figure 3B) (3.4 ± 0.5 versus 1.9 ± 0.4, n=7, p<0.01). Conversely, CLL cells had reduced calcium flux in response to anti-µ following transfection with an inhibitor of miR-155. The median increase in fluorescence intensity of calcium flux induced by anti-µ was significantly lower in cells transfected with inhibitor of miR-155 than in control-treated CLL cells (3.7 ± 0.4 versus 4.7 ± 0.5, n=6, p<0.01) (Figure 3C). Furthermore, we found that the median increase in calcium flux induced by treatment with anti-µ was significantly greater in ZAP-70-negative CLL cells with high-level-expression of miR-155 than in ZAP-70-negative CLL cells with low-level expression of miR-155 (2.5 ± 0.6 versus 1.0 ± 0.4 n=9, p<0.05) (Figure 3D).

**Intra-clonal Heterogeneity In CLL-Cell Expression Of miR-155**

We interrogated the PubMed GEO database on CLL cells from lymph node (LN), bone marrow (BM) or peripheral blood (PB). We found that expression of the precursor to miR-155, namely BIC (miR155HG), was significantly greater in CLL cells isolated from LN than in CLL cells isolated from the BM or PB of the same patient (Figure S5). On the other hand, the expression level of INPP5D, encoding SHIP1, appeared higher in CLL cells isolated from the blood than in CLL from the LN (Figure S6).
Recent studies have identified markers that can distinguish CLL cells that recently have exited from the LN from those that have been in the circulation longer and that presumably are poised to re-enter the LN tissue compartments. The former CLL cells express low-levels of CXCR4 (CXCR4\textsuperscript{dim}), but higher levels of CD5 (CD5\textsuperscript{bright}), relative to the latter, which are instead CXCR4\textsuperscript{bright}CD5\textsuperscript{dim} CLL cells. Prior studies demonstrated that CXCR4\textsuperscript{dim}CD5\textsuperscript{bright} CLL cells have higher proportions of cells that stain for the proliferation marker Ki-67 than CXCR4\textsuperscript{bright}CD5\textsuperscript{dim} CLL cells. Furthermore, gene expression analyses of isolated CLL subpopulations have revealed that CXCR4\textsuperscript{dim}CD5\textsuperscript{bright} CLL cells have higher levels of pro-proliferation and anti-apoptotic genes than CXCR4\textsuperscript{bright}CD5\textsuperscript{dim} CLL cells of the same patient.

We isolated blood CXCR4\textsuperscript{dim}CD5\textsuperscript{bright} CLL cells and CXCR4\textsuperscript{bright}CD5\textsuperscript{dim} CLL cells from the same patient and examined each subset for expression of \textit{miR-155} and SHIP1 and their capacity to respond to BCR ligation (Figure 4A). Expression of \textit{miR-155} was significantly higher in CXCR4\textsuperscript{dim}CD5\textsuperscript{bright} CLL cells than that in CXCR4\textsuperscript{bright}CD5\textsuperscript{dim} CLL cells (8.0 ± 4.5 versus 1.0 ± 0, n=4, \( p < 0.05 \)) (Figure 4B). On the other hand, expression of SHIP1 was significantly lower in CXCR4\textsuperscript{dim}CD5\textsuperscript{bright} CLL cells than that in CXCR4\textsuperscript{bright}CD5\textsuperscript{dim} CLL cells (Figure 4C). Furthermore, the median increase of calcium fluorescence intensity induced by anti-\( \mu \) was significantly greater in CXCR4\textsuperscript{dim}CD5\textsuperscript{bright} CLL cells than that in CXCR4\textsuperscript{bright}CD5\textsuperscript{dim} CLL cells (2.4 ± 0.4 versus 0.4 ± 0.1, n=10, \( p < 0.01 \)) (Figure 4D).

**The Effect Of CD154 Or BAFF On CLL-cell Expression Of \textit{miR-155}**

In LN, CLL cells interact with supportive cells, such as activated T-cells or Nurse-like cells, which can provide survival- or proliferation-inducing signals via the expression of members of the tumor necrosis factor (TNF) family of proteins (e.g. CD40-ligand or CD154), B-cell
activating factor (BAFF or CD257), or a proliferation inducing ligand (APRIL or CD256).\textsuperscript{34,35}

We examined whether CD154 or BAFF could upregulate CLL-cell expression of \textit{miR-155} and thereby influence the expression of SHIP1 and sensitivity to BCR ligation. CLL cells were stimulated with CD154 or BAFF for 24 or 48 hours \textit{in vitro} and then examined for \textit{miR-155}, SHIP1, and responsiveness to anti-\textmu. Treatment with CD154 or BAFF significantly upregulated expression of \textit{miR-155}, namely 2.7 ± 0.6 versus 1.0 ± 0 (n=3, \textit{p} <0.05) (Figure 5A) or 1.5 ± 0.1 versus 1.0 ± 0 (n=3, \textit{p} <0.05) (Figure S7A), respectively. Conversely, expression of SHIP1 was significantly lower in CLL cells treated with CD154 than in control cells (4.6 ± 0.7 versus 8.8 ± 1.2, n=6, \textit{p} <0.05) (Figure 5B) or in CLL cells treated with BAFF than in control cells (5.9 ± 1.0 versus 8.8 ± 1.2, n=6, \textit{p} <0.05) (Figure S7B). Furthermore, CD154 or BAFF enhanced the responsiveness of CLL cells to BCR ligation (Figure 5C, Figure S7C); the median increase of calcium fluorescence intensity induced by anti-\textmu was significantly greater in CLL cells treated with CD154 than in untreated cells (5.0 ± 1.1 versus 2.4 ± 0.4, n=9, \textit{p} <0.05) (Figure 5C) or in CLL cells treated with BAFF than in untreated cells (4.1 ± 2.2 versus 2.5 ± 1.9, n=4, \textit{p} <0.05) (Figure S7C). To examine whether the enhanced responsiveness to BCR ligation induced by CD154 was dependent upon induced expression of \textit{miR-155}, CLL cells were transfected with \textit{miR-155} inhibitor prior to treatment with CD154. We found that the median increase of anti-\textmu calcium fluorescence intensity induced by treatment with CD154 was significantly lower in CLL cells transfected with the \textit{miR-155} inhibitor than in cells transfected with control miRNAs (2.9 ± 1.3 versus 4.3 ± 1.2, n=4, \textit{p} <0.05) (Figure 5D).

We evaluated whether stimulation with CD154 could upregulate expression of \textit{miR-155} and thereby influence the expression of SHIP1 and responsiveness to BCR ligation in normal B-cells. Blood B-cells were isolated from normal donors (n= 3) and stimulated with CD154. After 24 or 48 hours of stimulation, we examined for changes in expression levels of \textit{miR-155}
and SHIP1, and in responsiveness to anti-μ. As noted for CLL cells, treatment with CD154
upregulated expression of miR-155, downregulated expression of SHIP1, and enhanced their
responsiveness to BCR ligation (Figure S8).
Discussion

Although prior studies identified an association between high-level expression of \( \text{miR-155} \) and expression of ZAP-70 or use of unmutated \( \text{IGHV} \), \(^{8,22,23,36,37} \) we found that this association was not absolute and that high-level expression of \( \text{miR-155} \) in CLL has additional independent prognostic value. We determined the optimal threshold for using \( \text{miR-155} \) (~2,550 copies CLL cell) to stratify patients in a training cohort of 86 patients into 2 subgroups with disparate progression tendencies by performing profile-likelihood analysis.\(^{38} \) We observed independent prognostic value of high-level \( \text{miR-155} \), over and above ZAP-70 and IGVH status. This observation was confirmed in an independent validation dataset of 181 patients. Although our threshold for defining high-level expression of \( \text{miR-155} \) does not take into account intra-clonal variation, it nonetheless could be useful for stratifying samples from patients for whom clinical outcome data might not be available. Importantly, defining high-level expression of \( \text{miR-155} \) in this manner allowed us to interrogate CLL cells with high versus low levels of \( \text{miR-155} \) for phenotypic and functional differences that may account in part for differences in clinical outcome.

We found that CLL cells with high-levels of \( \text{miR-155} \) expressed significantly lower levels of SHIP1 protein. SHIP1 is a phosphatase that is encoded by \( \text{INPP5D} \), which had been found to be a target for \( \text{miR-155} \) in cell types other than CLL.\(^{26} \) Although \( \text{INPP5D} \) encoding SHIP-1 is a known target gene for \( \text{miR-155} \), it has never been demonstrated that \( \text{INPP5D} \) levels inversely correlate with expression of \( \text{miR-155} \) in CLL cells. This is important, as each miRNA can have multiple different targets, which vary depending upon the cell type in which a given miRNA is expressed. High-level expression of an irrelevant gene that also was targeted by \( \text{miR-155} \), for example, could compete with \( \text{INPP5D} \) for its ability to be regulated by \( \text{miR-155} \). Indeed, prior studies by Loosner and colleagues identified a number of genes that could be targeted by
miR-155 in HEK293T cells. However, when they compared their results to previously identified miR-155 target proteins in other cell lines, they found major differences, demonstrating the cell-line specificity of microRNAs. Consistent with the notion that miR-155 regulates expression of INPP5D in CLL cells, we demonstrated through transfection studies that enhanced expression of miR-155 induces lower expression of INPP5D and that inhibition of miR-155 induced higher expression of INPP5D.

We also found that CLL cells with high-levels of miR-155 also were more responsive to surface-µ ligation than CLL cells with low-levels of miR-155. Prior studies found that CLL cells that lacked ZAP-70 generally experienced lower levels of activation following treatment with anti-µ. However, there were atypical cases that lacked expression of ZAP-70, but could be well-stimulated by treatment with anti-µ, suggesting that other factors could contribute to differences in sensitivity to BCR ligation. In the present study, we examined samples that did not express ZAP-70, but that had high versus low expression levels of miR-155. In such cases, the BCR signaling appeared to be associated with the expression-level of miR-155, suggesting that the relative expression of this microRNA could influence the relative proficiency of BCR-signaling in primary CLL cells. Transfecting CLL cells with miR-155 reduced expression of SHIP1 and enhanced the cells' sensitivity to surface-µ ligation. Conversely, transfection of CLL cells with a miR-155 inhibitor had the opposite effects. As enhanced signaling via the BCR has been implicated to contribute to disease progression in CLL, the capacity of miR-155 to influence the responsiveness to BCR-ligation may explain the noted association between high-levels of miR-155 and adverse outcome of patients with this disease.

The microRNA miR-155 also can regulate expression of other mRNAs encoding other proteins, such as the suppressor of cytokine signaling (SOCS)\textsuperscript{1,40,41} transforming growth factor (TGF) beta-1, TGF beta-2, or TGF beta-receptor type 2, which may play a role in tumor
invasion and/or metastasis. However, other than INPP5D, none of the known targets of miR-155 encode proteins that directly can influence BCR signaling. Similarly, SHIP1 expressed in hematopoietic cells could be repressed by over-expression of miR-155, which was associated with increased activation of the kinase AKT during the cellular response to LPS. In any case, our results here indicate that the relative levels of miR-155 could influence the proficiency of BCR-signaling in CLL.

Although the average leukemia-cell expression levels of miR-155 can be used to segregate patients at different relative risk for disease progression, the levels of miR-155 can vary between CLL cells of the same patient. Prior studies of Wang and colleagues observed high-level expression of miR155HG (BIC), the precursor to miR-155, in prolymphocytes and presumably CLL cells within the proliferation centers (PCs), which had high proportions of cells staining with the proliferation marker Ki-67. They noted that the cells within the PCs expressed high-levels of BIC/pre-miR-155 relative to the lymphocytes outside the PC via RNA in situ hybridization. The relative number BIC/pri-miR-155-positive cells varied from patient to patient, depending on the relative size and number of PCs present in the lymph node. We interrogated the GEO database (GSE21029) and noted that CLL cells in lymphoid tissues expressed higher levels of miR155HG, and apparently lower levels of INPP5D, than CLL cells in the peripheral blood. These data suggest that the levels of miR-155 can vary depending on where the CLL cells reside.

However, CLL cells are not fixed in their anatomic distribution, but rather re-circulate between blood, lymphoid, and marrow compartments. Prior studies indicated that a small proportion of CLL cells in the blood likely represent cells that have re-entered the circulation after exiting the lymphoid tissues. In particular, CD5brightCXCR4dim CLL cells in the blood represent newly-released cells from the tissue microenvironment, whereas the
CD5\textsuperscript{dim}CXCR4\textsuperscript{bright} CLL cells represent cells that may be about to re-enter the tissue microenvironment.\textsuperscript{33} In the present study, we found that the subgroup of blood CD5\textsuperscript{bright}CXCR4\textsuperscript{dim} CLL cells expressed higher levels of \textit{miR-155} and lower levels of SHIP1 protein, and were more sensitive to surface-\(\mu\) ligation than the “resting” subgroup of blood CD5\textsuperscript{dim}CXCR4\textsuperscript{bright} CLL cells (Figure 6). As such, this is the first study to define differences in the expression of \textit{miR-155} by CLL cells of the same patient and to find that this can result in functional differences in the leukemia-cells' responsiveness to BCR ligation.

We hypothesized that survival/stimulatory signals from accessory cells present in the leukemia-cell microenvironment, such as in the PCs of the LN, might contribute to the upregulation of \textit{miR-155} (Figure 6). To test this hypothesis we stimulated CLL cells with CD154 or BAFF, each of which could serve to activate NF-\(\kappa\)B.\textsuperscript{44,45} Prior studies found activation of NF-\(\kappa\)B could induce expression of \textit{BIC}, the precursor to \textit{miR-155}.\textsuperscript{46} However, in some B-cell lymphomas or B-cell lines (e.g. Ramos), NF-\(\kappa\)B–induced expression of \textit{BIC} did not necessarily cause increased expression of \textit{miR-155}, indicating that there might be two levels of regulation for generating mature \textit{miR-155}: one at the transcriptional level involving NF-\(\kappa\)B, and one at the processing level.\textsuperscript{46} The studies reported here demonstrate that exposure of CLL cells to factors that can activate NF-\(\kappa\)B significantly upregulated expression of mature \textit{miR-155}, suggesting that in CLL the control of \textit{miR-155} may predominately be at the level of \textit{BIC} transcription.

In any case, we found that the changes induced in the expression levels of \textit{miR-155} upon treatment with CD154 or BAFF had functional significance; such treatment significantly reduced expression of SHIP1 and enhanced the responsiveness to BCR ligation, effects that could be mitigated by transfection of CLL cells with an inhibitor to \textit{miR-155}. Similar effects of \textit{miR-155} also were noted for normal B-cells that were stimulated via CD40-ligation with CD154,
indicating that miR-155 might play a physiologic role in regulating the B-cell response to BCR ligation. Within the lymphoid-tissue microenvironment, T-cells or accessory cells may express factors, such as CD154 or BAFF/APRIL, which can enhance expression of miR-155. This in turn may serve as a rheostat that can enhance the signaling derived through BCR ligation (Figure 6). Conversely, attenuation in expression of miR-155 in CLL or normal B-cells when they circulate in the blood may allow for increased expression of SHIP1 and decreased responsiveness to BCR ligation. This could mitigate the risk for inadvertent stimulation by self- or foreign-antigen(s) of blood B-cells outside of lymphoid-tissue compartments, where they would not have the microenvironment and accessory cells necessary for antigen-stimulated B-cells to differentiate in an immune response to antigen. As such, miR-155 might play an important role in the physiology or normal B-cells and/or the pathophysiology of neoplastic B-cells.

These studies also have implications for development of new therapies that disrupt the capacity of CLL cells to home to tissue microenvironments or that inhibit the signaling from accessory cells within the microenvironment, thereby potentially interfering with the capacity of the microenvironment to upregulate miR-155. Agents that block the capacity of CLL cells to home or to engage with accessory cells in PCs might be expected to decrease the levels of miR-155 and increase expression of INPP5D. Monitoring for these changes in patients who are treated with such agents might become a useful surrogate marker for the capacity of the drug to cause diminished leukemia-cell stimulation in the lymphoid compartment. Also, agents that reduce expression of miR-155, or that up-regulate expression of SHIP1, might have therapeutic value in patients with CLL, particularly for those patients who have CLL cells with high levels of miR-155. Moreover, agents that inhibit leukemia-cell expression of miR-155 may have clinical activity in patients with this disease.
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Authorship Contribution: B.C. designed research, performed research, analyzed data, and wrote the paper; L.C. designed research, performed research, analyzed data, and wrote the paper; S. Z., M. M., J. F., J. Y., E.M.G., L.Z. and L.Z.R. performed research; L.B. and K. M. analyzed data and contributed to the manuscript; G.A.C., C.M.C. made scientific contributions; T.J.K designed research, analyzed data, and wrote the paper.

Conflict of Interest Disclosure: The authors declare no competing financial interests.
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Table 1. Characteristics of the Patients.

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<th>Characteristic</th>
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<tr>
<td>No. of patients</td>
<td>86</td>
<td>181</td>
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<tr>
<td>Male sex-no. of patients (%)</td>
<td>55 (64)</td>
<td>107 (59)</td>
</tr>
<tr>
<td>Rai Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-II</td>
<td>60 (70)</td>
<td>154 (85)</td>
</tr>
<tr>
<td>III-IV</td>
<td>9 (10)</td>
<td>6 (3)</td>
</tr>
<tr>
<td>No data</td>
<td>17 (20)</td>
<td>21 (12)</td>
</tr>
<tr>
<td>Age at diagnosis-yr</td>
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<td></td>
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<tr>
<td>Median</td>
<td>56</td>
<td>56</td>
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<tr>
<td>Range</td>
<td>34-78</td>
<td>34-81</td>
</tr>
<tr>
<td>ZAP70 Expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZAP-70-Negative (0% to 20%)</td>
<td>41 (48)</td>
<td>110 (61)</td>
</tr>
<tr>
<td>ZAP-70-Positive (&gt;20%)</td>
<td>45 (52)</td>
<td>71 (39)</td>
</tr>
<tr>
<td>IGVH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmutated (≥98% homology)</td>
<td>43 (50)</td>
<td>81 (45)</td>
</tr>
<tr>
<td>Mutated (&lt;98%homology)</td>
<td>43 (50)</td>
<td>100 (55)</td>
</tr>
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</table>
Table 2. Multivariable Cox regression analysis of treatment-free survival and overall survival (Cox proportional hazards regression model)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Training Dataset (qPCR data)</th>
<th>Validation Dataset (microarray data)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TFS</td>
<td>OS</td>
</tr>
<tr>
<td></td>
<td>HR 95% CI P</td>
<td>HR 95% CI P</td>
</tr>
<tr>
<td>miR-155 (high vs. low)</td>
<td>2.3 1.1-4.6 0.02</td>
<td>1.5 1.0-2.2 0.03</td>
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<tr>
<td>ZAP70 (pos vs. neg)</td>
<td>1.7 0.9-3.4 0.11</td>
<td>1.4 0.8-2.5 0.23</td>
</tr>
<tr>
<td>IGVH (unmut vs. mut)</td>
<td>1.8 0.9-3.6 0.09</td>
<td>2.6 1.4-4.7 0.002</td>
</tr>
</tbody>
</table>

| miR-155 (high vs. low) | 3.4 1.0-11.1 0.04 |
| ZAP70 (pos vs. neg)       | 3.2 0.9-11.0 0.07 |
| IGVH (unmut vs. mut)         | 7.0 1.8-27.8 0.006 |
Figure Legends

Figure 1. Relationship between expression levels of miR-155, ZAP-70, IGVH-mutation status, and TFS. (A) In the training dataset, Kaplan–Meier curves depicting the TFS probability over time from diagnosis of patients who were segregated into two groups (miR-155-Lo or miR-155-Hi) according to the relative amounts of miR-155 expressed by the blood CLL cells of each patient. (B-C) Kaplan–Meier curves depicting the TFS probability over time from diagnosis of patients segregated by miR-155 and ZAP-70 status (B), or miR-155 and IGVH-mutation status (C). (D) In the validation dataset, Kaplan–Meier curves depicting the TFS probability over time from diagnosis for patients who were segregated into two groups (miR-155-Lo or miR-155-Hi) based upon whether or not the blood CLL cells expressed miR-155 above the training-set-defined threshold for high miR-155HG. (E-F) In the validation dataset, Kaplan–Meier curves depicting the TFS probability over time from diagnosis for patients segregated by miR-155 and ZAP70 status (E) or miR-155 and IGVH status (F). Statistical significance was determined by log rank test (p<0.05). p values for the comparisons between subgroups are indicated below the graph for panels B, C, E, and F.

Figure 2. Expression of SHIP1 protein in CLL cells that expressed high versus low levels of miR-155. (A) Expression of SHIP1 in CLL cells that expressed high (n= 21) or low (n=24) levels of miR-155, as indicated at the bottom of the histogram. The height of each column corresponds to the mean MFIR of cells stained for SHIP1 in each subgroup. Error bars indicate the S.D. of the mean. Statistical significance was determined by unpaired Student’s t test (p<0.05). (B) Expression of SHIP1 in representative CLL samples, CLL#1 (Rai stage 1 at diagnosis, mutated IGHV, ZAP-70 negative, Δ peak anti-µ induced MFIR = 7) or CLL#2 (Rai stage 2 at diagnosis, mutated IGHV, ZAP-70 positive, Δ peak anti-µ induced MFIR = 32),
following transfection with mimic-ct (control microRNA) or miR-155 microRNA, as indicated at the bottom of each panel. (C) Expression of SHIP1 in representative CLL samples, CLL#3 (Rai stage 2 at diagnosis, unmutated IGHV, ZAP-70-positive, Δ peak anti-µ induced MFIR = 4.2) or CLL#4 (Rai stage 0 at diagnosis, mutated IGHV, ZAP-70-negative, Δ peak anti-µ induced MFIR = 1.4), following transfection with oligo-ct (control microRNA) or miR-155 inhibitor, as indicated at the bottom of each panel. In (B) or (C) data respectively are presented from immunoblot analyses (top panels) or flow cytometry (bottom panels). After the immunoblots were probed with anti-SHIP1, they were stripped and probed with anti-β-actin to monitor the uniformity of protein loading. The numbers between the immunoblot panels provide to the ratios of the density of the SHIP1 band relative to that of the β-actin band, normalized with respect to the ratio observed in control-treated samples. The histograms in the bottom panel depict the mean MFIR of CLL cells stained for SHIP1 following transfection with (B) mimic-ct or miR-155 mimic or (C) oligo-ct or miR-155 inhibitor, as indicated at the bottom of each histogram. Statistical significance was determined by paired Student’s t test (p<0.05).

**Figure 3. Anti-µ induced calcium flux in CLL cells that express high versus low levels of miR-155.** (A) The height of each column in the histogram describes the increase in fluorescence intensity after anti-µ stimulation of CLL cells that expressed high-miR-155 versus low-miR-155, as indicated at the bottom of the histogram. (B) Anti-µ-induced calcium mobilization in CLL cells following transfection with mimic-ct (top graph) or miR-155 (lower graph). The relative mean fluorescence intensity in intracellular calcium is plotted as a function of time. The arrow labeled “IgM” indicates the time at which the anti-µ was added to the cells. In the histogram at the bottom, the height of each column corresponds to the mean increase of fluorescence intensity after anti-µ stimulation for samples transfected with either
mimic-ct or miR-155 microRNA, as indicated at the bottom by a “+”. (C) Anti-µ-induced calcium mobilization in CLL cells following transfection with oligo-ct or miR-155 inhibitor. The relative mean fluorescence intensity in intracellular calcium is plotted as in panel B. The height of each column in the bottom histogram corresponds to the increase of fluorescence intensity after anti-µ stimulation for samples respectively transfected with either control-ct or miR-155 inhibitor, as indicated at the bottom by a “+”. (D) The height of each column in the histogram describes the increase in fluorescence intensity after anti-µ stimulation of ZAP-70 negative CLL cells with high-miR-155 or low-miR-155, as indicated at the bottom of the histogram. Statistical significance was determined by the unpaired Student’s t test (p<0.05).

Figure 4. Expression of miR-155, SHIP1, and sensitivity to anti-µ in subpopulations of CLL cells isolated from the blood mononuclear cells of the same patient. (A) Flow cytometric analyses of representative unsorted CLL cells (left), isolated CD5brightCXCR4dim CLL cells (upper right), or isolated CD5dimCXCR4bright CLL cells (lower right). Contour plots (10% probability) depict the fluorescence of CLL cells stained with fluorochrome-conjugated mAbs specific for human CD5 (y-axis) or human CXCR4 (x-axis). (B) Expression of miR-155 in CLL cells that expressed high-levels of CD5 and low-levels of CXCR4, or low-levels of CD5 and high-levels of CXCR4. The height of each column in the histogram indicates the fold-increase of miR-155 copy number of sorted CD5brightCXCR4dim cells relative to that of sorted CD5dimCXCR4bright cells, as indicated at the bottom. Statistical significance was determined by paired Student’s t test (p<0.05). (C) Expression of SHIP1 in sorted CD5brightCXCR4dim or CD5dimCXCR4bright CLL cells of representative samples (CLL1 (left two lanes) or CLL2 (right two lanes)), as indicated at the bottom. Probing for β-actin was used to monitor for equal loading of sample lysates. (D) Anti-µ-induced calcium mobilization in CLL cells that expressed
high-levels of CD5 and low-levels of CXCR4, or low-levels of CD5 and high-levels of CXCR4. The relative mean fluorescence intensity in intracellular calcium is plotted in Figure 3B and 3C for each cell subset; The height of each column in the histogram on the right corresponds to the increase of fluorescence intensity after anti-µ stimulation for sorted CD5^{bright}CXCR4^{dim} or CD5^{dim}CXCR4^{bright} cells, as indicated at the bottom. Statistical significance was determined by unpaired Student’s t test (p<0.05).

Figure 5. Stimulation of CLL cells with CD154 can induce expression \textit{miR-155}, down-regulation of SHIP1, and enhanced BCR signaling  (A) Expression of \textit{miR-155} in CLL cells without (-) or with (+) CD154 stimulation. The height of each column in the histogram corresponds to the fold increase in expression of \textit{miR-155} in CLL cells stimulated with CD154 (+) relative to that in CLL cells that had not been so stimulated (-), as indicated at the bottom of the histogram. We determined statistical significance using the paired Student’s t test (p<0.05). (B) Expression of SHIP1 in CLL cells without (-) or with (+) CD154 stimulation, as indicated at the bottom of the histogram. The height of each column corresponds to the mean MFIR for SHIP1. (C) Anti-µ-induced calcium mobilization in CLL cells without (Ct, top panel) or with (CD154, lower panel) CD154 stimulation. The relative mean fluorescence intensity in intracellular calcium is plotted as a function of time. The arrow labeled “IgM” indicates the time at which the anti-µ was added to the cells (left panel). The histogram to the right depicts the mean increase of fluorescence intensity of CLL cells following stimulation with anti-µ without (-) or with (+) prior stimulation with CD154, as indicated at the bottom of the histogram. (D) Anti-µ-induced calcium mobilization in CLL cells with (“+”) or without (“−”) CD154 stimulation after the cells were transfected with a control oligonucleotide (oligo-ct) or \textit{miR-155} inhibitor. The height of each column in the histogram corresponds to the mean increase of fluorescence
intensity following treatment with anti-µ of CLL cells. We determined statistical significance using one-way ANOVAs (p<0.05).

**Figure 6. Model of the phenotype of CLL cells exiting the lymph node into the blood or exiting the blood into the LN**  
Within the proliferation centers (PC) of the lymph node, CLL cells are stimulated via factors in the microenvironment (e.g. CD154 or BAFF/APRIL), where there is upregulation of *miR-155* and down-regulation of *INPP5D*. The CLL cells that recently have exited the LN express relatively low-levels of CXCR4 (*CXCR4<sup>Dim</sup>*), high levels of CD5 (*CD5<sup>Bright</sup>*), high-levels of *miR-155* (*miR-155<sup>High</sup>), and low-levels of SHIP1 (*SHIP1<sup>Low</sup>), and have high-responsiveness to BCR ligation with anti-µ (*BCR-signaling<sup>High</sup>*). Conversely, the cells that may exit the blood for the lymph node are *CXCR4<sup>Bright</sup>*-*CD5<sup>Dim</sup>* and have relatively low levels of *miR-155* (*miR-155<sup>Low</sup>), high levels of SHIP1 (*SHIP1<sup>High</sup>), and relatively low-responsiveness to BCR ligation with anti-µ (*BCR-signaling<sup>Low</sup>*). The arrows indicate the direction of trafficking from the blood to the lymph node and then back to the blood.
Figure 2

A

B

C

- Actin

- mimic-ct

- miR-155 mimic

- miR-155 inhibitor

- oligo-ct

p<0.01

p<0.05

p<0.05
Figure 4

A

CD19+ CLL cells

Log CD5 Fluorescence

Log CXCR4 Fluorescence

Analysis of sorted cells subpopulations

CD5<sup>bright</sup> CXCR4<sup>dim</sup>

CD5<sup>dim</sup> CXCR4<sup>bright</sup>

B

Relative miR-155 expression

P < 0.05

C

CLL#1  CLL#2

SHIP1

β-actin

D

Relative [Ca<sup>2+</sup>]^

IgM

CD5<sup>bright</sup> CXCR4<sup>dim</sup>

CD5<sup>dim</sup> CXCR4<sup>bright</sup>

Δ Peak Fluorescence

Δ Intensity

P < 0.01
Figure 6

Lymph Node

Proliferation Center (PC)

$\uparrow$ miR155

$\downarrow$ INPP5D

CD5$^{\text{Dim}}$

CXCR4$^{\text{Bright}}$

miR155$^{\text{Low}}$

SHIP1$^{\text{High}}$

BCR-Signaling $^{\text{Low}}$

CD5$^{\text{Bright}}$

CXCR4$^{\text{Dim}}$

miR155$^{\text{High}}$

SHIP1$^{\text{Low}}$

BCR-Signaling $^{\text{High}}$

Blood Vessel
MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia

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